DEVELOPMENT AND METABOLISM OF COPPER-DEFICIENT OAT PLANTS

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(Accepted for publication 11th March, 1946.)

In starving leaves of grasses, Wood, Cruickshank and Kuchel (1943) pointed out that rate of protein hydrolysis and rate of oxidation of ascorbic acid were apparently related. Attention was focussed upon copper-deficient plants, since it seemed that these might provide means of varying rates of ascorbic acid oxidation through their varying copper contents.

Riceman and Donald (1938) recognized copper-deficiency in plants grown on highly calcareous sands in the South East of South Australia, and it has since been shown (Riceman and Anderson, 1943) that similar soils are widespread in South Australia. Piper (1942) has made a careful study of the effects of varying amounts of copper supplied to plants grown in water culture. Riceman, Donald and Evans (1940) have presented data showing relations between yield and varying copper supply of plants grown in sand culture and in the field. Piper and Walkley (1943) have described the copper contents of plants of agricultural importance at time of flowering. References to previous work on copper-deficiency are summarized in these papers.

It has been made clear from the work cited above that most varieties of oats (*Avena sativa*) are especially susceptible to copper-deficiency; the characteristic symptoms of deficiency being (a) "wither-tip", i.e. white colour and failure to unroll of newly developing leaves, (b) "white heads", i.e. failure to produce grain and in severe cases failure of panicle emergence, (c) excessive tillering.

Data for final yield of dry matter in relation to differential manurial treatments frequently give few indications of the processes occurring within the plant and in this paper a study, of an exploratory kind, has been made of the changes which occur in the development, chemical composition and metabolism of different plant organs at different stages in the life-cycle of oat plants when these were supplied with different amounts of copper.

EXPERIMENTAL.

Culture and Treatments.

A pure line of oat plants (*Avena sativa* var. Mulga) were grown in Robe sand in glazed earthenware pots in a glasshouse. The latter soil has been described by Thomas *et al.* (1938), and is an alkaline, highly calcareous sand derived from marine shell fragments. The soil for the experiment described here was obtained from an area carrying natural depauperate grassland and known to be copper-deficient. The oven-dried soil contained 0·010 p.c. N.

Each pot contained 3 kg. sand. Six oat grains per pot were sown on 26th April, 1945, thinned to four even plants per pot on day 20 and thinned to two plants per pot after the first harvest on day 57.

The pots were divided into four series according to the treatments designed for them. The four treatments applied were:

Treatment A. Basal nutrients, but without Cu, i.e. + N — Cu.
Treatment B. Fully manured, i.e. + N + Cu.
Treatment C. Basal nutrients, but without N and Cu, i.e. — N — Cu.
Treatment D. Basal nutrients, but without N, i.e. — N + Cu.

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[1] Research Assistant under Federal Government Research Grant to Universities.

The fully-manured series (Treatment B) received the following nutrients in gm. per pot: $Ca(H_2PO_4)_2$, 0·5 gm.; $NaNO_3$, 0·5 gm.; KCl, 0·4 gm.; $MgSO_4.7H_2O$, 0·14 gm.; Fe_2Cl_6, 0·015 gm.; $MnSO_4.4H_2O$, 0·005 gm.; $ZnSO_4$, 0·005 gm.; $CuSO_4.5H_2O$, 0·040 gm.; $NaMoO_4.2H_2O$, 0·001 gm. On the 97th day after sowing symptoms of N-deficiency appeared and an additional 0·5 gm. $NaNO_3$ per pot was added to Treatments A and B. Total dressings over surface of pot were equivalent to 2 cwt./acre of $Ca(H_2PO_4)_2$ and $NaNO_3$; 10 lb./acre of Fe_2Cl_6; 3½ lb./acre of $MnSO_4$ and $ZnSO_4$ and 14 lb./acre of $CuSO_4$.

Plants of Treatment A received the same nutrients as in Treatment B, but with omission of $CuSO_4$.

In Treatments C and D, $NaNO_3$ was omitted from all pots; $CuSO_4$ was omitted in Treatment C but plants of Treatment D received 0·040 gm. $CuSO_4$ per pot.

$NaNO_3$, KCl and $MgSO_4$ solutions were freed from Cu by extraction with dithizone and elimination of excess of the latter. $Ca(H_2PO_4)_2$ was prepared from $CaCl_2$, and KH_2PO_4 solutions, both previously freed from Cu by dithizone extraction. Fe_2Cl_6, $MnSO_4$ and $ZnSO_4$ solutions were prepared from salts twice re-crystallized from A.R. grade chemicals.

Throughout the experiment pots were maintained at 60 p.c. of the water retaining capacity of the soil by twice-weekly additions of water and weekly weighings. Cu-free water was used throughout, prepared by re-distillation from Pyrex glass stills.

Harvests.

Appropriate census data were collected at fortnightly intervals throughout the life-cycle. At frequent intervals the developing inflorescence was dissected out from plants grown in spare pots and camera lucida drawings made. Measurements of cell size in leaves were made at intervals.

Harvests were made on the following days after planting: *Harvest I*, 57 days; *Harvest II*, 95 days; *Harvest III*, 139 days; *Harvest IV*, 193 days. Twenty-eight plants from each treatment were harvested at Harvest I, and fourteen plants from each treatment at each of the other harvests. The third harvest was omitted in Treatments C and D owing to insufficient material.

On each harvest day, where appropriate, the following separations of plant parts were made: living leaves; dead leaves; roots, stems, inflorescence (i.e. panicle cut at node bearing the lowest spikelets); grain. At the first three harvests appropriate parts in any one treatment were bulked, but at the final harvest plant parts from each individual plant were kept separate to enable statistical analysis to be made. Immediately after separation, the various portions of the plant were weighed and samples of these dried to constant weight at 90° C. in a current of air.

At Harvest II aliquot samples of living leaves from each treatment were placed in black containers and subjected to starvation at 24° C. according to the procedure described by Wood, Cruickshank and Kuchel (1943).

At Harvest III aliquot samples of living leaves were ground with sand and a known volume of the homogenized material placed in solution containing excess ascorbic acid. Rate of disappearance of ascorbic acid was determined by titration at intervals.

Analytical Methods.

Copper[2] was determined by Piper's (1942) modification of the method of Sylvester and Lampitt after wet digestion of the plant material, colour comparisons being made in a spectrophotometer.

Protein–N was determined by micro-Kjeldahl after extraction of dried leaf material and precipitation of protein at pH 4·5 according to the method described by Wood *et al.* (1943).

Crude fibre was determined by the method of Henneberg and the fraction lignin *plus* cutin by treating the crude fibre with 70 p.c. H_2SO_4 according to the procedure of Ost and Wilkening. An estimate of the amount of *lignin* was obtained by treating the lignin *plus* cutin fraction with H_2O_2 and ammonia according to the method of Konig. These methods are described by Klein (1932).

Ascorbic acid was determined by Lugg's (1942) modification of the dichlorphenol-indophenol titration.

RESULTS.

Census Data and Symptoms.

Treatments A and B. Census data for Treatments A and B are presented graphically in Fig. 1. From the start the number of tillers in the Cu-deficient plants (Treatment A) was greater than in Treatment B. On addition of further nitrate on day 95 the number of tillers was increased in both treatments; many of these tillers subsequently died, but in the Cu-deficient series this loss was made good by numbers of late secondary tillers which developed after day 150. At Harvest IV the number of panicle-bearing tillers was significantly greater in Treatment B than in Treatment A (see Table 3).

There were more leaves per plant in Cu-deficient plants, a consequence of the greater number of tillers. However, the most striking feature of total leaf number was the fact that after exsertion

[2] We are greatly indebted to D. W. Dewey of the Division of Biochemistry and General Nutrition, C.S.I.R., who carried out most of the copper analyses by courtesy of the Director of the Division.

of the panicle about day 130 no further increase in leaf-number occurred in Treatment B; on the other hand, in Cu-deficient plants production of leaves continued, chiefly on late secondary tillers, up to the time of Harvest IV. After exsertion of the inflorescence the number of living leaves decreased more rapidly in Treatment B than in Treatment A; however the rate of production of dead leaves was approximately the same in both treatments.

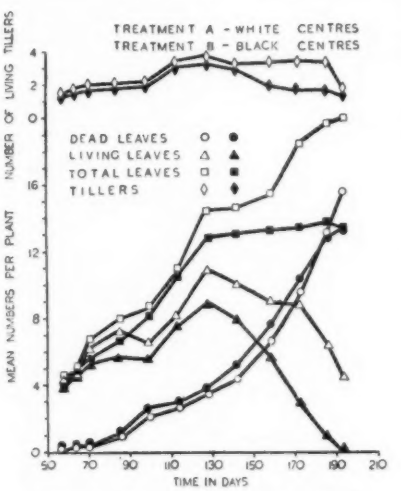

Fig. 1. Census data for Treatments A and B.

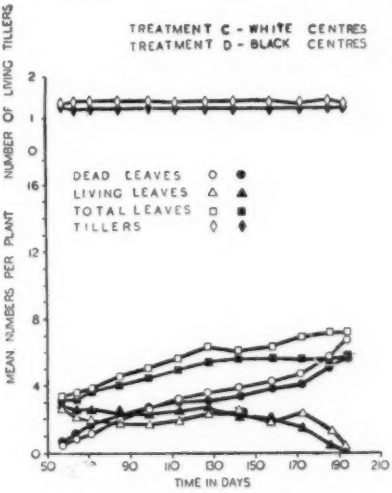

Fig. 2. Census data for Treatments C and D.

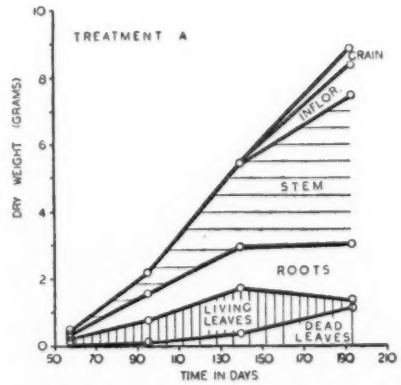

Fig. 3. Dry weights of plant parts plotted additively. Treatment A.

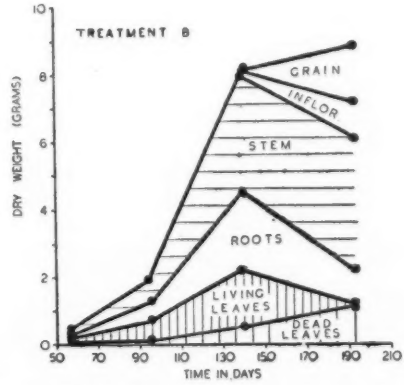

Fig. 4. Dry weights of plant parts plotted additively. Treatment B.

Externally, plants of both treatments appeared similar up to about day 116. Between days 116–140 some of the leaves in the Cu-deficient series became limp and on all plants in this treatment emerging leaf-tips were first yellow, then white and the leaf died before unrolling. This phase was relatively transient; thereafter fairly vigorous growth of late secondary tillers occurred and on these the wither-tip symptoms characteristic of Cu-deficiency were rare. After day 140 day temperatures rose markedly and we are inclined to ascribe recovery of the plants to greater uptake or greater availability of Cu in the soil with rising temperatures. Inflorescences were peeping on day 130 and anthesis commenced about day 145 in both series.

At Harvest IV the plants of the two treatments were similar in appearance to those described and figured by Riceman *et al.* (1940), and Piper (1942). Plants of Treatment B were normal tall plants with dry straw, well developed panicles and plump grain. Plants of Treatment A were shorter, with many tillers, with some green leaves, straw green at the base and panicles poorly developed with many "white heads", i.e. blanched dead spikelets, especially towards the top of the inflorescence: grain development was poor and many spikelets were empty, but individual grains appeared normal.

Treatments C and D. In the N-deficient plants of Treatments C and D the most striking difference between treatments throughout the vegetative phase was in number of tillers (Fig. 2). All plants of Treatment D possessed one tiller only; the Cu-deficient plants averaged 1·5 tillers per plant; this feature appeared about day 40 and was maintained throughout the life of the plants.

The number of living leaves remained approximately constant throughout the greater part of the life-cycle, due to N-starvation, new leaves appearing as the older ones lost protein during senescence. No symptoms of Cu-deficiency were apparent in the leaves; however when the panicles appeared, in every plant in both treatments the top half of the panicle consisted of white dead spikelets. The appearance of this symptom in the series receiving copper as well as in the Cu-deficient series is doubtless due to the very small root systems possessed by these nitrogen-deficient plants (see Table 1). As in Treatments A and B the production of green, living spikelets in the lower parts of the panicle is probably correlated with greater availability of copper with rising temperatures. Flowering and anthesis occurred at the same time in both treatments and about a week later than in Treatments A and B.

Dry Weight Data.

Data for the mean dry weights of various plant parts at Harvest IV are set out in Table 1.

TABLE 1.

Mean dry weights per plant of plant parts at Harvest IV (gm.).

(Means significantly different at 5 p.c. point shown in italics).

Treatment	A.	B.	C.	D.
Leaves: living	0·27	—	—	—
dead	1·43	1·43	0·42	0·39
Stems	5·52	4·86	1·17	0·89
Inflorescence	1·11	1·33	0·34	0·32
Grain	0·60	*2·03*	0·57	0·63
Roots	*2·12*	1·37	0·34	0·31
Total	11·04	11·02	2·84	2·54

There was no significant difference between mean total dry weights of plants in Treatments A and B. The weight of grain in Treatment B was significantly greater than in Treatment A, whilst the mean root weight of Treatment A was significantly greater than in Treatment B; both differences were significant at the 1 p.c. point. Comparing Treatments C and D, in no case were the differences in dry weights of any plant part significant.

Comparing the *plus*-N with the *minus*-N series, the mean dry weight of each portion of the plant was significantly greater in the former series than in the latter, except in the case of the grain in Treatment A which was not significantly greater than that in Treatments C and D.

Greater insight into dry weight changes is gained by consideration of the weights of plant parts at various growth stages. Data for the four harvests are plotted cumulatively for Treatment A (Fig. 3) and Treatment B (Fig. 4).

Up to and including Harvest III differences in mean dry weight per plant of various plant organs were not significantly different. At Harvest III maximum leaf number had been attained in Treatment B and inflorescences were peeping in both treatments. At Harvest III mean dry weights of leaves, roots and stems were greater in Treatment B than in the Cu-deficient Treatment A.

The characteristic differences between the two treatments appear by comparing the changes which occurred between Harvests III and IV in each treatment.

In Treatment A there was no significant change in leaf dry weight between Harvests III and IV, but an *increase* in dry weight occurred in root and stem. Inflorescence and grain dry weights were small. It is clear that increase in stem dry weight between the two harvests was the main factor causing approximate equality of total dry weights at maturity in the two treatments.

On the other hand, in Treatment B there was a *decrease* in dry weight of leaves and roots between Harvests III and IV, whilst changes in stem dry weight were probably not significant. There was a large increase in grain dry weight.

Summing up, it may be said that the dry weight changes during ontogeny in Treatment B are characteristic of normal oat plants (cf. Williams, 1936), whilst those in Treatment A are characteristic of "topped" plants, i.e. plants in which the inflorescence has been suppressed or removed and in which consequently vegetative growth continues (cf. dry weights of "topped" and untopped tobacco plants described by Petrie *et al.*, 1939).

Distribution of Protein in the Plants.

At each harvest the amounts of protein-N in different plant parts of Treatment A and B were determined. These are given on a relative basis (gm. per 100 gm. dry weight) in Table 2 and absolute amounts of protein-N are plotted cumulatively in Figs. 5 and 6. On a relative basis,

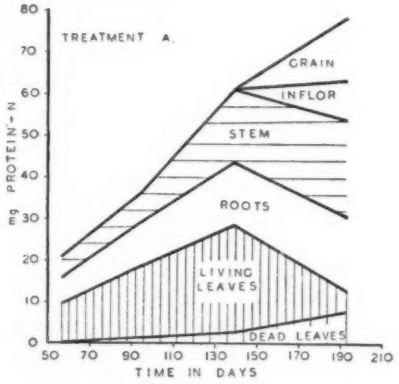

Fig. 5. Absolute protein content of plant parts plotted additively. Treatment A.

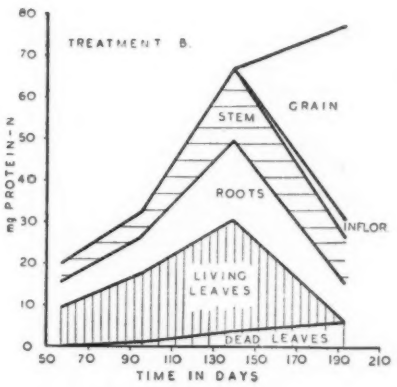

Fig. 6. Absolute protein content of plant parts plotted additively. Treatment B.

percentage of protein-N decreases with time in leaves, stems and roots, following the well known ontogenetic drift reported by Richards and Templeman (1926), Petrie (1937) and Williams (1938). The percentage protein in all these parts is greater at late harvests in Treatment A than in Treatment B.

TABLE 2.

Protein-N (percentage dry weight) in different plant organs.

Treatment	A.				B.			
Harvest	I	II	III	IV	I	II	III	IV
Leaves: living	4·00	2·22	1·73	1·78	3·80	2·20	1·35	—
dead	—	0·85	0·64	0·54	—	0·84	0·54	0·43
Roots	2·90	1·10	0·88	0·84	2·91	1·06	0·65	0·66
Stems	3·50	0·85	0·57	0·42	3·40	0·77	0·40	0·23
Inflorescence	—	—	0·57	0·86	—	—	0·40	0·34
Total straw	3·80	1·48	0·91	0·55	3·67	1·42	0·70	0·29
Grain	—	—	—	2·56	—	—	—	2·31

Figs. 5 and 6 wherein absolute amounts of protein-N are plotted bring out more clearly the differences between the two treatments. In Treatment A (Fig. 5) between Harvests III and IV there occurred a decrease in amount of leaf-protein, but increase in amounts of stem and

root protein. Increase in amounts of inflorescence and grain protein was small. On the other hand, in Treatment B, between Harvests III and IV there was a marked decrease in amount of protein in leaves, roots and stem and a very large increase in grain-protein.

These protein analyses confirm the conclusions arrived at from the dry weight data. Plants of Treatment B behaved as normal plants; during maturation of the grain protein hydrolysis occurred in vegetative organs, the products were transported to the grain and laid down as grain protein. Plants of Treatment A behaved as "topped" plants; little grain protein was formed and there was an increase in amounts of root and stem protein. The increase in number of late secondary tillers is a result of suppression of the inflorescence.

Development of the Inflorescence.

In view of the conclusions reached in the last sections special interest is attached to the development of the inflorescence.

Characteristics relating to the inflorescence at Harvest IV are given in Table 3.

TABLE 3.

Characteristics of inflorescence (means per plant) at Harvest IV.

(Means significantly different at 5 p.c. point shown in italics).

Treatment	A.	B.	C.	D.
Mean height (cm.)	15·7	*21·1*	12·8	13·0
No. panicle-bearing tillers	1·8	*2·5*	1·0	1·0
No. sterile tillers	*3·6*	0	0·4	0
No. green spikelets per panicle	16·6	*23·3*	13·2	15·9
Dry weight of grain	0·60	*2·03*	0·57	0·63

At intervals from an early stage (62 days) development of the inflorescence was studied by careful dissection from spare plants in each series. Development of organs and tissues in the flower and spikelet were studied and drawn under camera lucida, and measurements made of lengths of inflorescence and of spikelets when the latter formed. Stages of development of the panicle are shown in Figs. 7a and 7b; length of inflorescence in Fig. 8 and length of spikelets in Fig. 9.

Pertinent descriptions regarding development are summarized below:

Day 62. Primary branch initials of inflorescence present in all cases. Very few initials of secondary branches. Same stage in all treatments. Differences such as those between (1) and (4) often occur in plants of the same pot. Leaf initials present at base of inflorescence. *Vide* (1)–(4), Fig. 7a.

Day 72. Secondary branch initials prominent. Initials of flower, lemma and empty glumes present in apical spikelets in all treatments. Much same stage in all treatments; D slightly less advanced than A and B, C at much same stage at D but less extensive development owing to deficiencies. *Vide* (4)–(7), Fig. 7a.

Day 84. Flower initials present in all spikelets. Anther initials present in apical flowers. Much same stage of development in all treatments with same provisos as on day 72. *Vide* (9)–(12), Fig. 7b.

Day 95. Anther initials prominent in upper flowers and developing in lower ones. Much same stage in Treatments A and B; slightly less extensive development in C and D. *Vide* (13), Fig. 7b for Treatment B.

Day 113. In Treatment A and B: Length of spikelet increases regularly from the base to apex of panicle. In apical spikelet, awn, which grows faster than the empty glumes, was just projecting beyond glumes. In lower spikelets glumes shorter than in apical spikelets and awns not projecting. In lower flower of each spikelet stamens well developed and lobes distinct. Pistil developing ridge which grows up and over top. *Vide* (14) and (15), Fig. 7b for Treatment A.

At about this stage the elongation of the empty glumes was most marked. Although the corresponding flowers in each spikelet of the upper half of the panicle were in similar developmental stages, the glumes of the apical spikelet had elongated to 6 or 8 times their length 18 days previously, and completely enclosed the flowers. Glumes of upper spikelets were considerably longer than in lower spikelets.

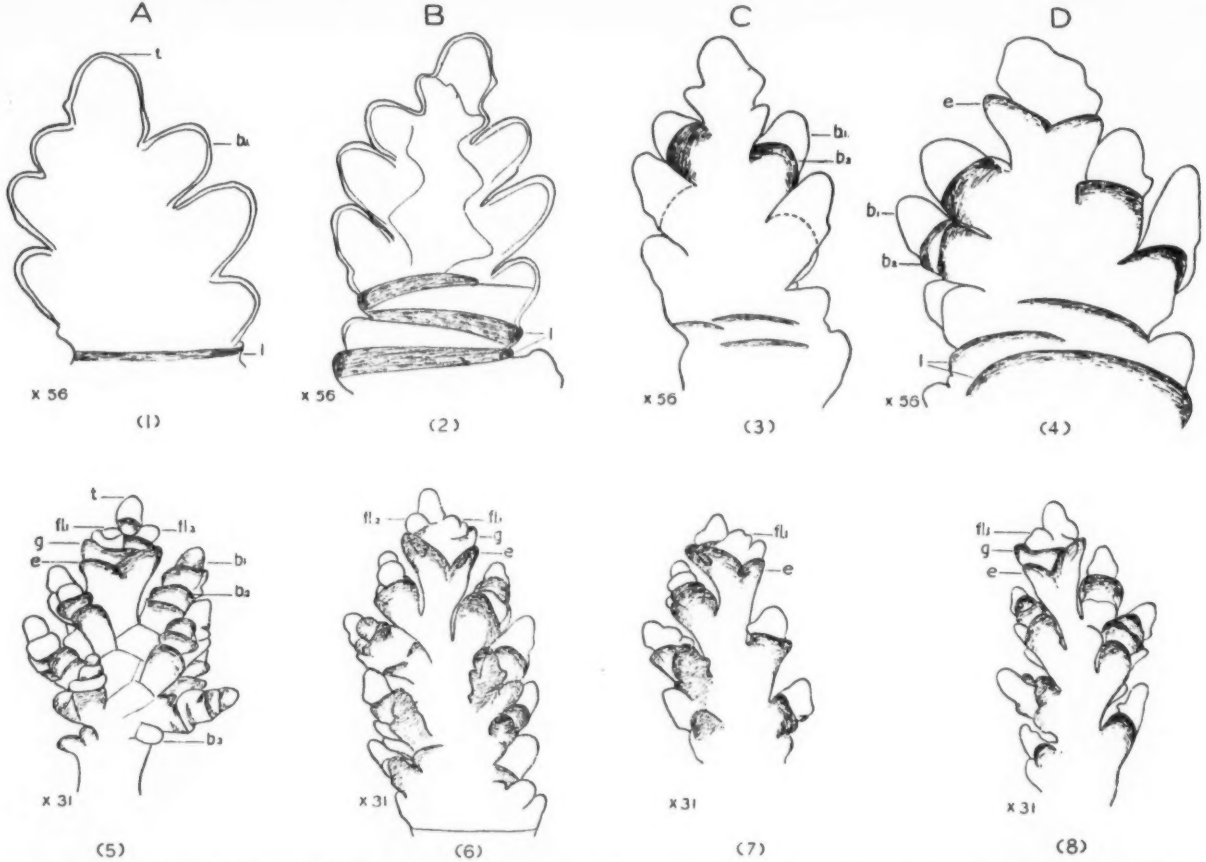

Fig. 7a. (1)–(4): Early stages of panicle formation in the four Treatments A, B, C and D respectively, showing primary branch initials and leaf initials. Differences between the treatments not significant. Age, 62 days. (5)–(8): Secondary branch initials prominent, with empty glumes and flower 1 developing. A and B (5, 6) slightly more advanced than C and D (7, 8). Age, 72 days. t, tip of main axis; b1, b2, b3, primary, secondary and tertiary branch initials; l, leaf initial; e, empty glumes; fl1, fl2, first and second flowers of the spikelet; g, lemma.

A
B
C
D
an, fl3
fl3
fl1
g
e
x 23
(9)
fl2
fl3
fl1
g
e
x 23
(10)
an, fl2
g
g, fl3
an, fl1
g
x 31
fl1
fl2
e
x 23
(11)
e
e
x 23
(12)
fl2
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x 26
(15)
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This stage is clearly one of rapid increase in length of the empty glumes and is followed only slightly later by growth of the awn which soon outgrows the glumes and appears above them. From day 113 onwards the branches of the panicle elongated greatly; in the 13 days following, the panicle of Treatment B increased in height by about 20 cm.

In Treatment D awns not yet projecting, but anthers lobed and pistils developing as above.

Treatment C: spikelets less developed; stamens distinctly 4-lobed, but small; pistil less developed than in D.

Day 126. In Treatments A and B, lowest flower in apical spikelet well developed; hairs on ovary, styles erect; other flowers in apical spikelets less well developed. Both treatments at same stage of development but *differences in lengths of spikelets apparent in Treatment A* (see Fig. 9). Normal tapetum and pollen mother cells in anthers. In Treatment D, much same stage as above but development slower; Treatment C, as in D but less advanced. *Vide* (16), Fig. 7b for Treatment B.

Day. 148. In living spikelets of A and B, lower flower fully developed, post-fertilization, anthers dehisced and filaments broken; styles fully expanded and covered with papillae, pollen grains with pollen tubes on styles. *Vide* (17) Fig. 7b for Treatment A. Second flower of spikelets almost mature. Many spikelets in Treatment A dead, with shrivelled and bleached glumes, enclosing shrivelled remains of flowers developed to stage on day 126. *Vide* (16) Fig. 7b. Anthers and styles developed to stage above but bleached and shrivelled. Death apparently occurred suddenly. In Treatments C and D whole of upper half of panicle consisted of dead spikelets; lower spikelets alive in both treatments, but less advanced than in Treatments A and B.

Day 168. In Treatment B, in apical spikelets ovary enlarged, scutellum and embryo visible; outer glumes hardening. In lower spikelets glumes still soft and grains milky. In Treatment A, many dead spikelets, living spikelets in same stage as lower spikelets of B. Sharp distinction between dead and withered spikelets and normal fully developed ones; no intermediate stages of dead spikelets.

Treatment C and D less advanced than A and B, grain developing but small and soft.

From these data it is clear that up to day 126 the same stage of development of flowers was reached in all treatments, though somewhat less extensive in Treatments C and D owing to N-deficiency. At this time the spikelets were about 20 mm. long, the awn was scarcely exserted beyond the glumes, pistils were normal and styles erect and microscopic examination showed normal tapetum and pollen mother cells within the anthers. It is also clear that development of the upper spikelets in the inflorescence was more advanced than in lower ones and that the lower flower in each spikelet was more developed than the upper flower.

The general detail of panicle and spikelet development as found in our experiment is in full agreement with that described for oats by Bonnett (1937).

Between days 126 and 148 many of the spikelets in the Cu-deficient series did not develop beyond this stage and apparently died quite suddenly.

In the variety of oats (Mulga) used in our experiment, two flowers (only one of which bears an awn) are normally developed in each spikelet, although rudiments of a third are normally present and very occasionally a fourth. In the Cu-deficient series, one fully developed flower was often present in each spikelet, the second having ceased to develop at an early stage. This indicates that only a partial Cu-deficiency was present, there being sufficient Cu present to complete development of one of the normal two flowers.

In this connection the data of Figs. 8 and 9 for inflorescence length and spikelet-length respectively are significant. Fig. 8 shows that the inflorescence elongated greatly between days

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EXPLANATION OF FIG. 7b.

Fig. 7b. (9)–(12): Treatments A, B, C and D respectively. Anther initials present in lower flowers of the apical and upper spikelets in all treatments, branches of panicle commencing to elongate. Little difference between treatments, except slight retarding in deficient ones. Apical flower, with empty glumes removed, is shown enlarged to right of (10). Age, 84 days. (13): Treatment B, age 95 days: anther initials developed in flower 1 of all except lower spikelets, and in flowers 2 and 3 of apical spikelet. This stage is just prior to elongation of panicle branches. (14): Treatment A, age 113 days. Apical spikelet, showing relative dimension of empty glumes, awn and flower. (15): Enlargement of flowers and awn base of (14); lemma removed in flower 1; anthers developing in first three flowers, rudiments of a fourth flower present. (16): Treatment B, age 126 days; flower 1 of apical spikelet, with lemma removed; lodicules, styles, anthers well developed. (17): Mature oat flower (Treatment A, age 148 days); anthers dehisced, post fertilization. fl1, fl2, fl3, fl4, first to fourth flowers of spikelet; e, empty glumes; g, lemma; an, anthers; gp, growing point; a, awn; p, palea; s, styles; st, stigma; lo, lodicules.

110 and 130 and Fig. 9 that arrest of development of spikelets in Cu-deficient series occurred at the same time. Reference to the section on census data and symptoms shows that this also was the time at which the characteristic symptoms of Cu-deficiency occurred, *viz.* wither-tip and loss of turgor of leaves.

The evidence therefore is strong that at this time insufficient copper reached the developing flower organs presumably to form a copper complex of some kind in their cells. These flowers subsequently died and converted the plants in effect to "topped" plants. The development of fertile flowers in the lower parts of the inflorescence of Cu-deficient plants was simultaneous with the partial cessation of Cu-deficiency symptoms in the leaves. It will be shown in the section dealing with distribution of copper that analyses for copper substantiate these conclusions.

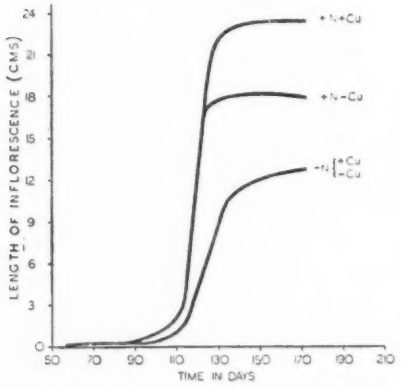

Fig. 8. Length of inflorescence of main tiller at different times.

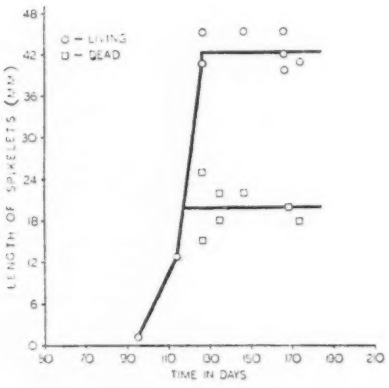

Fig. 9. Length of spikelets at different times.

The conclusions reached here provide a rational basis for the experiments of Riceman *et al.* (1940), at Teeluk in the South-East of South Australia, where very late application of Cu (about 4 months after sowing) to oats showing severe Cu-deficiency symptoms, caused abundant panicle formation and good grain development.

Leaves and Stem.

Anatomical examination of leaves and stem. Ford (1940) has compared anatomical differences in leaves and stems of Cu-deficient oat plants with normal controls; the plants were grown in acid, Cu-deficient, podsolized sands from Western Australia. Ford found that in stems of normal plants there was a thickened epidermis, a zone of lignified cells in the cortex, thick lignified cells in the bundle sheath and thick-walled cells in the outer pith. In Cu-deficient plants all the cells remained thin-walled and unlignified. The leaf thickness was greater in normal plants than in Cu-deficient plants owing to greater number of cells in cross-section; motor-cells were poorly developed in Cu-deficient plants and sclerenchyma was less abundant than in normal plants.

In the experiments described in this paper, the lengths of all living leaves in each series were measured at intervals and also counts and measurements of cell size were made. These were carried out under standard conditions. Comparable leaves of the same age and position on the stem were taken in each treatment. Transverse sections were cut two to three inches from the ligule, and measurements made of leaf thickness, number of cells in cross-section between upper and lower epidermis, and dimensions of central and side motor-cells. These measurements were made in two definite positions in all cases since the leaf becomes thinner towards the edges; one series of measurements was made between the 6th and 7th veins on either side of the midrib and another series between the 9th and 10th veins on either side of the midrib. Statistical examination of mean values was performed. Mean values are given in Table 4.

TABLE 4.

Mean dimensions of various attributes of leaves.

(Means of 12 observations).

	Treatment.	Height of plants (cm.).	Length of leaves (cm.).	6–7 vein. Leaf thickness (μ).	6–7 vein. No. of cells thick.	6–7 vein. Size of motor cells (μ) Central.	6–7 vein. Size of motor cells (μ) Side.	9–10 vein. Leaf thickness (μ).	9–10 vein. No. of cells thick.	9–10 vein. Size of motor cells (μ) Central.	9–10 vein. Size of motor cells (μ) Side.
Day 104	A	n.d.	28·5	214	3·8	55·7 × 48·0	42·4 × 27·3	174	3·3	50·2 × 40·2	38·8 × 27·8
	B	n.d.	31·4	221	4·0	54·9 × 43·0	42·2 × 27·8	182	3·7	48·9 × 35·8	38·6 × 27·6
	C	n.d.	29·8	150	3·6	47·4 × 37·7	36·6 × 22·0	134	3·1	40·0 × 31·8	31·5 × 21·4
	D	n.d.	29·8	174	3·7	43·8 × 32·8	34·3 × 20·2	150	3·4	43·5 × 30·6	34·9 × 23·6
Day 132	A	80	26·3	276	4·0	59·5 × 46·7					
	B	99	28·7	275	4·1	61·1 × 46·9					
Day 168	A	110	24·0	203	3·0	57·5 × 47·7	45·8 × 31·8	174	3·0	52·1 × 42·5	41·6 × 28·3
	B	135	24·4	212	3·3	57·0 × 45·2	44·1 × 28·1	187	3·0	49·9 × 34·0	42·5 × 31·5
	C	100	n.d.	163	3·3	45·8 × 38·2	38·6 × 26·5	148	3·0	44·5 × 36·3	39·1 × 27·2
	D	94	n.d.	150	3·0	43·9 × 35·4	35·2 × 24·0	144	3·0	40·7 × 33·3	35·0 × 25·0

(For significant differences see text.)

Leaves of Treatment B were significantly longer (5 p.c. point) than those of the Cu-deficient Treatment A on days 104 and 132, but on day 168 there was no significant difference. This finding is in harmony with the onset of Cu-deficiency symptoms in the developing inflorescence at the former time and later recovery.

At no time was there a significant difference between Treatments A and B in leaf thickness, number of cells in cross-section or in size of motor-cells. Similarly there was no significant difference in these attributes between Treatments C and D.

However, N-deficiency had a marked effect, although the number of cells in cross-section of the leaf was the same in all treatments; leaf thickness and size of cells was significantly lower in the *minus*-N plants than in the *plus*-N plants. This difference in cell size is probably correlated with protein contents and consequent difference in degree of hydration of the cells; for example, on day 95 (Harvest II) just prior to the examination on day 104, the protein contents, expressed as percentage of dry weight, of Treatments A, B, C and D were 2·22, 2·20, 1·81 and 1·60 p.c. respectively.

On day 132 the leaves examined in Treatment A were immediately below and partly enclosing leaves which were white and failed to unroll (i.e. below those exhibiting characteristic wither-tip symptoms), yet these leaves showed no significant differences in cell size from those of normal plants.

TABLE 5.

Amounts of crude fibre and lignin in the stems.

	Treatment.	Crude fibre (p.c. dry weight).	Lignin (p.c. crude fibre).
Harvest I	A	24·0	2·1
	B	24·9	2·6
	C	27·0	2·5
	D	27·5	2·9
Harvest II	A	24·2	2·4
	B	24·4	2·8
	C	27·0	3·0
	D	26·4	3·4
Harvest III	A	27·2	2·8
	B	27·1	2·8
Harvest IV	A	37·0	2·8
	B	38·0	2·8

No differences in degree of lignification could be detected in any of the leaves. There was insufficient leaf material to carry out analyses at all harvests, but on day 139 (Harvest IV) analyses for crude fibre and lignin were carried out in living leaves of Treatment A and B. Leaves of Treatments A and B contained 18·0 p.c. and 18·4 p.c. crude fibre respectively expressed as percentage of dry weight; and 1·3 p.c. and 1·4 p.c. lignin respectively expressed as percentage of crude fibre. The small differences between these sets of figures are within limits of error of the method of analysis.

Analysis of the stems for crude fibre and lignin were carried out at each harvest; these are shown in Table 5.

Differences in crude fibre and lignin contents between *plus*-Cu and *minus*-Cu plants are not significant, but *minus*-N plants contained a greater percentage of crude fibre than *plus*-N plants, probably owing to the greater relative amount of carbohydrate available for wall formation in the N-deficient plants.

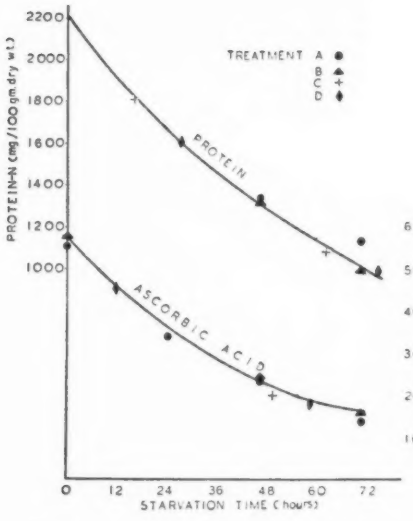

Fig. 10. Amounts of protein and ascorbic acid at different starvation times.

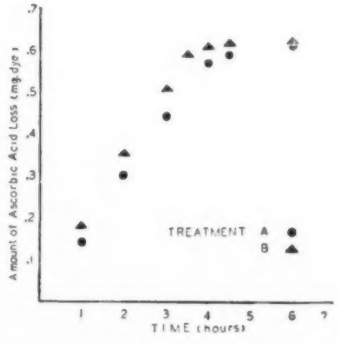

Fig. 11. Rate of loss of ascorbic acid. Treatments A and B.

It will be seen that the results described here are at variance with those of Ford. We are unable to account for the differences, although those described by Ford for the stem are seen if the stems compared are not of the same age. However, from the plates provided, it is evident that the Cu-deficient plants examined by Ford differed markedly from ours in that they were dwarfed, very poorly developed and showed much greater signs of Cu-deficiency than those in our experiments.

In our experiments, leaves showing symptoms of Cu-deficiency exhibited well defined changes. The characteristic feature was lysis of cell contents and collapse of all chloroplast-containing cells; veins, epidermal cells and parenchymatous bundle sheaths of veins were intact. Lignification was well developed in unrolled white leaves. Collapse of all chlorenchyma tissue resulted in rolling of the leaves; all other thickened tissues remained normal except the motor cells which became buckled; the upper epidermis, having a fairly thick cuticle remained intact. Buckling of the motor cells was due entirely to loss of support from the turgid chlorenchyma tissue, not to any decrease in amount of cutinization. Similar lysis and collapse of mesophyll cells was described by Reed (1939) in leaves of Cu-deficient tomatoes.

Summing up, in the experiments described in this paper, there were no significant differences between Cu-deficient and normal plants in leaf thickness, size of cells in leaf nor in degree of lignification of cells in leaves and stems. Onset of Cu-deficiency symptoms was sudden and caused lysis and collapse of chlorenchyma cells. This in turn causes (a) white colour to the leaves, (b)

rolling of leaves following collapse of motor cells, these giving the symptom of "wither-tip".

Behaviour of leaves during starvation. At Harvest II replicate samples of living leaves from each treatment were placed in dark containers and starved at 24° C. as described in a previous section. Samples were withdrawn for analysis at the commencement of starvation and on the second and third days of starvation; at each time ascorbic acid was determined in an aliquot of the living leaves from each treatment; the remaining leaves were dried at 90° C. in an air current, their dry weight determined and subsequently protein-N determined. All amounts were expressed as percentage of original dry weight. The initial protein contents were not the same in all treatments; treatments A, B, C and D contained respectively 2·22, 2·20, 1·81 and 1·60 p.c. protein-N on a dry weight basis. Protein-N contents in Treatments A and B were plotted against time and a curve drawn; the initial values for Treatments C and D were plotted at the appropriate place on this curve which was taken as zero time for these treatments. Results are given in Fig. 10. Values for ascorbic acid were treated in a similar manner and are also given in Fig. 10.

From these curves the amounts of change in both protein-N and ascorbic acid at 12-hour intervals were read off and are tabulated in Table 6.

TABLE 6.

Amount of change in protein and ascorbic acid at various times during starvation.

Time (hours).	Amount of change (mg./100 gm. dry weight). Protein-N.	Ascorbic acid.	Ratio. Protein-N. / Ascorbic acid.
0	0	0	
12	340	130	2·62
24	620	230	2·70
36	800	300	2·66
48	940	360	2·62
60	1,100	400	2·74
72	1,200	410	2·78

From these data it appears that the different manurial treatments produced no effect on the rate of breakdown of protein in leaves during starvation at this harvest. Table 6 indicates the close connection between rate of protein hydrolysis and rate of loss of ascorbic acid which had been noted previously by Wood, Cruickshank and Kuchel (1943) and Wood, Mercer and Pedlow (1944).

Rate of ascorbic acid loss. Since ascorbic oxidase is a Cu-protein enzyme it might be expected to decrease in amount in Cu-deficient leaves. At Harvest III, the uppermost, fully expanded living leaves in Treatments A and B were harvested separately; the leaves of Treatment A were immediately below white unrolled leaves with characteristic Cu-deficient symptoms. Duplicate lots of 1 gm. fresh weight of these leaves were ground with clean quartz sand and washed into a test-mixer till the total volume was 20 ml. in each case. 4 ml. of 0·6 p.c. ascorbic acid solution were added so that the final solution contained 0·1 p.c. ascorbic acid. This solution was placed in a plugged Erlenmeyer flask, gently shaken on a mechanical shaker and samples withdrawn for analysis at intervals. Immediately after mixing and at the sampling times 2 ml. samples were withdrawn and added to a 10 ml. flask containing 2 ml. 16 p.c. HPO_3; the volume was made up to 10 ml., filtered through a dry filter and 5 ml. taken for ascorbic acid determination.

The amount of change in this aliquot (i.e. the amount of ascorbic acid which had disappeared) measured in terms of ml. of dye is given in Fig. 11 for Treatment A and B.

It is apparent that differences in rate of ascorbic acid loss between the two treatments are probably not significant, even though the leaves of Treatment A were immediately below leaves showing extreme symptoms of Cu-deficiency.

Summing up our investigations on living leaves in normal and Cu-deficient plants it may be said that living leaves of the latter did not differ significantly from those of normal plants in anatomical or in metabolic characteristics up to the time of wither-tip symptoms.

Distribution of Copper in the Plants.

At each harvest the amounts of copper were determined in plant parts of Treatments A and B; copper contents of the roots could not be determined owing to likelihood of contamination by soil particles. Amounts of copper on a relative basis (p.p.m.) are given in Table 7 and absolute amounts of copper in each plant part, plotted cumulatively are given in Figs. 12 and 13.

TABLE 7.

Copper (parts per million on dry weight basis) in different plant organs.

Treatment	A.				B.			
Harvest	I	II	III	IV	I	II	III	IV
Leaves: living	5·16	2·58	4·00	3·44	5·18	2·94	2·70	—
dead	—	7·70	6·00	7·53	—	8·15	7·70	7·14
Stems	4·00	1·20	1·65	0·88	4·00	1·29	1·23	0·91
Inflorescence	—	—	—	1·33	—	—	—	1·29
Total straw	4·80	2·13	2·77	2·11	4·84	2·52	2·09	2·15
Grain	—	—	—	1·76	—	—	—	1·87

Data hitherto published for amounts of copper in plants have referred only to total straw and to grain. The amounts of copper in the straw at different growth stages in our experiment corresponded closely with those described by Piper and Walkley (1943). In living leaves the percentage copper showed decrease with age of the plant; this decrease was marked in Treatment B, where other sinks, especially the developing inflorescence, compete with leaves for copper. In Treatment A, the percentage amount of copper in the living leaves reached its lowest value at Harvest II and thereafter was followed by an increase and later still decreased in amount; the low value at Harvest II is associated with Cu-deficiency symptoms in leaves and death of the developing spikelets; as pointed out previously arrest of these symptoms and also increase in percentage copper were associated with rising external temperatures.

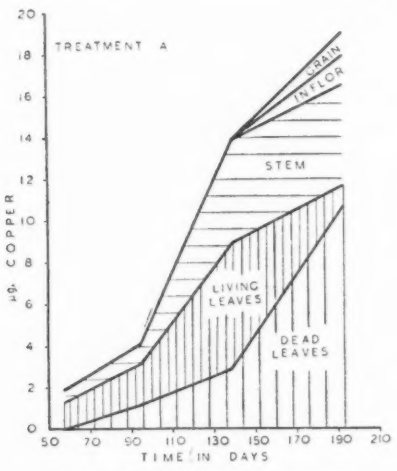

Fig. 12. Absolute copper content of plant parts plotted additively. Treatment A.

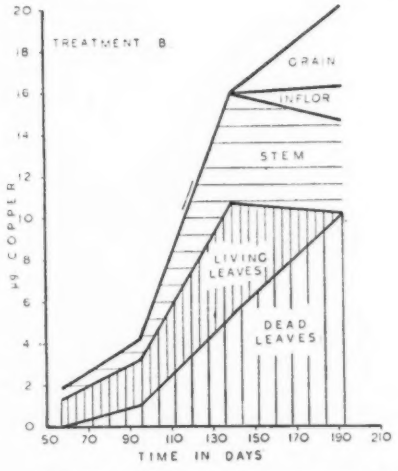

Fig. 13. Absolute copper content of plant parts plotted additively. Treatment B.

In general, the percentage decrease in copper with age resembled that of protein-N, but the most striking feature of copper distribution was the high percentage of copper in the dead leaves, suggesting that, unlike nitrogen, copper is relatively immobile in the leaves. This feature will be discussed more fully below.

Percentage amounts of copper in stems decreased with time in both treatments.

Data for the absolute amounts of copper in different plant parts (Figs. 12 and 13) show a continuous uptake of copper in the aerial portions of the plant throughout the life-cycle; at Harvest III the total uptake of copper was less in Treatment A than in Treament B, but at Harvest IV approximately the same amounts of copper had been taken up by plants of both Treatments.

In Treatment B there was no loss of copper from the leaves between Harvests III and IV, the total amounts of copper in these organs remaining the same at both harvests. In Treatment A there was a continuous uptake of copper by the leaves. In this respect leaf copper behaves differently from leaf protein.

Particular interest is attached to the copper in the dead leaves. In Treatment B the amount of copper in the dead leaves at Harvest IV is equal to the sum of that present in living and dead leaves at Harvest III; in Treatment A the amount of copper in the dead leaves is greater than that of the sum of living and dead leaves at Harvest III, but here continued uptake of copper occurred after Harvest III.

The evidence is strong therefore that copper is immobilized in the leaves and is not transported during senescence.

This conclusion was checked as follows: In Treatment B at Harvest I the plants each possessed six leaves (not all fully expanded) on two tillers; at Harvest III these same leaves were all dead and they were collected separately. In both these lots of leaves the copper content was determined and also the crude fibre content. Dry weight cannot be used as a basis for comparison owing to losses of protein and carbohydrates from the dead leaves; crude fibre content of living leaves however showed little variation; leaves at Harvest I contained 18·0 p.c. crude fibre and those at Harvest IV 18·4 p.c. both on a dry weight basis. The living leaves at Harvest I contained 18·0 p.c. crude fibre and 5·2 p.p.m. of copper; the same leaves dead at Harvest III contained 24·5 p.c. crude fibre and 7·7 p.p.m. copper; this is equivalent to $7{\cdot}7 \times \frac{18}{24{\cdot}5}$, i.e. 5·6 p.p.m. on the original dry weight basis. Taking into account the fact that the leaves were not fully expanded at Harvest I the calculated value of 5·6 p.p.m. is sufficiently close to the found value of 5·2 p.p.m. to show that no loss of copper had occurred from these leaves.

In the stems, Figs. 12 and 13 show that there was no change in amounts of copper between Harvests III and IV in both Treatments A and B; however, since the dry weight of the stem increased in Treatment A over this period it is apparent that some re-distribution of copper occurred within the stem.

It is clear therefore that the copper present in the grain was not derived from the leaves or stems, but was derived either from the roots or by absorption from the soil. Piper (1942) showed clearly in oats grown in water culture that if seedlings were grown for at least a month in solutions containing 100 μg Cu and then transferred to Cu-free solutions then sufficient copper was taken up by the plant to allow normal growth and grain formation; much of this copper must have been stored in roots and stem, since few leaves are present at this stage. Whether some copper may be stored and transported from leaves when the plant is presented with a luxury supply of copper is not known. Copper is not readily available in the alkaline Robe soil (pH 8·2) since both Riceman *et al.* (1940), and Piper (1942) have found that heavy dressings of $CuSO_4$, up to 2 cwt. per acre caused little increase in amount of copper in the plant over those receiving 14–28 lb. $CuSO_4$ per acre.

The form in which copper occurs in the living and dead leaves is not yet known; it is possible that a Cu-protein complex is formed. In Treatment B, apparently, uptake of copper from the soil was sufficient to form a Cu-complex in the cells throughout the life-cycle; in Treatment A cessation or retardation of Cu-uptake, coupled with its immobility in the leaves was sufficient to cause the sudden appearance of Cu-deficiency symptoms in the leaves and death of developing spikelets.

SUMMARY.

An experiment is described wherein oats (*Avena sativa*, var. Mulga) were grown on a copper-deficient South Australian soil. Four treatments were applied: with and without additional copper, and with and without additional nitrogen. All N-deficient plants, as well as those receiving no additional copper, showed Cu-deficiency symptoms, presumably owing to poor root development. High-N, high Cu plants were normal. In Cu-deficient plants characteristic symptoms were white leaves which failed to unroll, loss of turgor and poor grain development with many small dead spikelets.

Dry weight and protein data for plant parts at each harvest showed that plus-Cu plants were normal whilst Cu-deficient plants behaved as "topped" plants. Dissection of the inflorescence at intervals showed that spikelets died suddenly in Cu-deficient plants when about 20 mm. long, anther and ovary development up to this stage being normal.

Onset of Cu-deficiency symptoms were sudden. Comparison of leaves from normal plants and leaves immediately below others exhibiting deficiency symptoms showed no anatomical (cell size; degree of lignification) or metabolic differences (rate of protein hydrolysis; rate of ascorbic acid loss). Characteristic white Cu-deficient leaves showed chloroplast disintegration, lysis of cell contents and collapse of all chlorenchyma cells.

Copper analysis of plant parts at different growth stages showed that copper content on a dry weight basis decreased with age. Copper uptake from the soil was continuous throughout the life-cycle. Unlike nitrogen, copper is immobile in the leaves, the amount present in living leaves remaining behind in dead leaves. Insufficient uptake of copper at the time of spikelet formation coupled with its immobility in leaves was sufficient to account for the deficiency symptoms observed. There is evidence that uptake of copper by the plants increased with rising external temperatures.

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ALLOXAN DIABETES IN THE SHEEP

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(Accepted for publication 4th March, 1946.)

It has been suggested (Marston, 1939, 1946) that, should the ruminant derive most of its energy requirements from the lower fatty acids which result from the fermentation of cellulose and related carbohydrates, sugar may assume a rôle of subsidiary importance in its intermediary metabolism.

The concentration of glucose in the blood of normal sheep and goats is approximately half that of man and other animals. This low concentration is not appreciably diminished after several days of fasting and massive doses of insulin are needed to lower it materially below the normal level of between 40 and 50 mg. per 100 ml. When this is reduced to approximately one-third, the usual symptoms of insulin shock do not supervene; the animal does not convulse but, after a period of apathy, subsides to coma (Cutler, 1934; Strand, Anderson and Allcroft, 1934).

Complete removal of the pancreas from the ruminant is extremely difficult to achieve but the production of a "mild" form of diabetes in goats by incomplete pancreatectomy has been described (Lukens, 1938). The recent discovery that intravenous injection of alloxan results in specific necrosis of the islet tissue of the pancreas (Shaw Dunn, Sheehan and McLetchie, 1943), and the reports by other workers (Bailey and Bailey, 1943; Goldner and Gomori, 1943a, 1943b) that the symptoms typical of diabetes mellitus may be induced by this means in other animals, led to the following experimental study of alloxan diabetes in the sheep; there was no record of this lesion in ruminants and we required diabetic sheep for the study of the fat and carbohydrate metabolism of this animal.

EXPERIMENTAL METHODS.

Six Merino and six Merino-Corriedale ewes of varying ages ranging from 2 to 7 years were used. For the first six experiments the alloxan was prepared by oxidation of uric acid (Hartman, Sheppard and Nightingale, 1943), and in the others a high-grade commercial preparation was used. In each case the dose was dissolved in about 50 ml. of distilled water and injected into the jugular vein. Doses ranged between 90 and 200 mg. per kg. body weight. The animals were confined in metabolism cages (Marston, 1935) throughout the period of the experiment. The food and water intake and the urine output were measured. The rations consisted of a mixture of two parts of wheaten hay chaff and one part of lucerne chaff, and on occasions fresh green lucerne or green oats were offered: the former will be referred to as the dry feed and the latter as the green feed. When the animals had been rendered diabetic about 50 gm. of sodium bicarbonate were added to the ration. For animal 637, 25 gm. of a salt mixture (sodium chloride 25 p.c., calcium carbonate 15 p.c., magnesium sulphate 15 p.c., potassium chloride 5 p.c., dicalcium phosphate 20 p.c., sodium sulphate 20 p.c.) were added to the feed each day. Water was allowed *ad lib.* The animals were weighed periodically.

After establishing the blood-sugar level for some days a sample was taken just prior to the injection of the alloxan; subsequently, samples were collected either hourly or two-hourly over the next 16–18 hours and then once or twice daily throughout the experimental period. The urinary output was collected at 9–9.30 a.m. each day, tested qualitatively for the presence of albumin, for sugar and for ketone bodies, and the specific gravity and pH determined. Aliquot samples were taken for quantitive estimation of total ketone bodies by the gravimetric method of Van Slyke (Peters and Van Slyke, 1932a) and of reducing sugars by the titration method of Benedict (1911). Cole's method (Cole, 1933) was chosen for the last two experiments on account of its convenience and the much clearer titration end-point. Analyses by either method were sufficiently in agreement to justify the use of the more convenient means. Blood sugar was estimated by the Somogyi modification of the Shaffer-Hartman method (Peters and Van Slyke, 1932b).

At the end of the period of observation, the animals were killed and an autopsy performed at which portions of various tissues were taken into fixatives for histological examination. Kidney, liver and adrenal slices were fixed in Zenker or Zenker-Formol (10 parts of Formol to 90 parts Zenker stock) for 24 hours and portions of pancreatic tissue from various sites were fixed in Bouin, Zenker, and Zenker-Formol for 6–8 hours. The tissue removed from the pancreas was not more than 3 mm. thick and after a half-hour in the fixative was cut to about 1–2 mm. thick. The material was embedded in paraffin and sectioned at 4–5μ. The kidney, liver and adrenal were stained with routine haematoxylin and eosin; the chrome-haematoxylin phloxin method described by Gomori (1941) and the Azan-Mallory method (Gomori, 1939) were used for the pancreas.

Attempts were made to assay the insulin content of the pancreas taken from two diabetic animals (Nos. 49 and 203) and seven normal animals, 4 ewes aged 5 years and 3 ewes aged 2–3 years. The mouse convulsion method (Trevan and Book, 1926) was used for some of the assays; there were insufficient mice available for critical estimations so the cross-over technique with rabbits (Marks, 1926) was adopted. The tissue was extracted by the procedure of Scott and Fisher (1938).

RESULTS.

The general reaction of each animal to the particular dose of alloxan administered is shown in Table I. From this it can be seen that 3 animals, Nos. 203, 637 and 49, were maintained in a diabetic condition for some time. Six of the animals (Nos. 861, 24, 42, 6, 9, 258) received a dose that was sufficiently toxic to lead to complications which were the cause of death within about a week. All of these animals, with the exception of No. 24 which lived only about a day, had a sustained hyperglycaemia after the first 24 hours but most of them exhibited, in addition, symptoms of acute kidney damage which may well have been responsible for the fatal consequence. Two animals, Nos. 119 and 623, were slaughtered 13 hours after the injection of alloxan in order to obtain material for histological examination. At this time their behaviour was normal and there were no signs of toxic effects. They had exhibited a preliminary and transient hyperglycaemia of about 240 mg. per 100 ml., followed by a hypoglycaemia of about 20 mg. per 100 ml.—the usual reaction to alloxan. When they were slaughtered the blood sugar concentration of each was between 20 and 30 mg. per 100 ml. In another animal, No. 1, in which the dose was insufficient a slight preliminary rise and fall in the blood sugar concentration (maximum 100 mg. per 100 ml. and minimum 37 mg. per 100 ml.), indicated that some reaction had occurred, but after the first 24 hours a normal level was resumed and this persisted for 7 days until the animal was killed for autopsy. No macroscopic lesions were found. The responses of animals Nos. 203, 637 and 49, together with the pertinent biochemical data are shown in Figs. 1, 2 and 3. Figs. 4 and 5 depict the course of events in animals Nos. 6 and 9; these two were chosen to illustrate the typical behaviour of the cases which suffered the fatal effects of extra-pancreatic damage.

With the exception of animal No. 1 and those three animals that did not survive for the first 24 hours, all responded similarly. Following alloxan injection an initial and transitory hyperglycaemia (the blood sugar level rose to about 240–300 mg. per 100 ml.) supervened in about 3–5 hours. This was immediately followed by a fall to a hypoglycaemic level of 20–30 mg. per 100 ml.; the lowest level in this phase was reached about 12–14 hours after the injection. A sustained hyperglycaemia then developed and glycosuria and ketonuria, characteristic of true diabetes, were evident. An average of between 50 and 70 gm. sugar, and approximately 12 gm. total acetone bodies, expressed as acetone, were excreted each day. The urine output was usually markedly increased above a normal value of 400–1,000 ml. each day; on most days a volume of 3,000–4,000 was voided. The reaction of the urine was generally about pH 8·0–8·5 owing to the rather massive quantities of fixed base that were consumed in the rations.

In those animals which received sufficient alloxan to produce more general damage, the course of events was somewhat similar; they all exhibited the triphasic blood sugar response, and subsequently developed glycosuria and ketonuria, but usually after a few days they refused food. On some occasions the volume of urine decreased markedly and albumin was excreted. This was noticeable in Nos. 861, 6, 9 and 258. The blood sugar concentration often rose to very high levels; in No. 42 it reached a level of 1,000 mg. per 100 ml. on the fourth day after the injection. The animal was then obviously ill, signs of marked respiratory distress developed and when in a state of collapse it was killed. The blood sugar level of No. 861 rose to 840 mg. per 100 ml. on the third day after injection and the animal died on the seventh day after exhibiting albuminuria and haematuria, signs of acute kidney damage. It had consumed only 70 gm. of dry feed during the period. Insulin injected into this animal after the fourth day failed to reduce the blood sugar below 200 mg. per 100 ml. and did not improve the condition. Insulin therapy of the three animals, Nos. 203, 637 and 49, resulted in a marked reduction in the blood sugar level although some difficulty was encountered in maintaining a normal level for No. 203. The symptoms of diabetes were materially alleviated by insulin treatment and the glycosuria and ketonuria were reduced to about a normal level.

Post-mortem examination of those animals that were maintained diabetic for a relatively long time (Nos. 203, 637 and 49) showed little, if any, gross abnormality. The nutritive condition as indicated by a fairly good cover of omental and perirenal fat was reasonably good. Animal No. 49 was an exception. This animal had a yellowish granular material in the calices of the left kidney and the bladder was empty except for a few drops of thick yellowish suspension.

TABLE 1.

Animal. No.	Age. yrs.	Body wt. kg.	Dose of alloxan. gm.	Survival period. days.	Condition of animal.	Cause of death.
1	6	35	3·3	7	Not diabetic. In excellent general condition.	Killed for autopsy. No abnormality detected.
203	3	42	4·2	47	Diabetic. Condition generally fair throughout with some improvement following insulin therapy.	Ditto.
637	7	39	3·5	52	Diabetic. Condition generally fair throughout.	Ditto.
49	4½	36	3·6	14	Diabetic. Apathetic from 3rd day but general condition fair until 12th–14th day when weakness developed.	Killed for autopsy. No gross macroscopic abnormality detected.
119	4	32	3·1	½	Appeared well throughout day.	Killed 13 hours after injection for autopsy.
623	7	35	3·1	½	Ditto.	Ditto.
861	7	30	6·0	7	Diabetic. Marked decline in general condition from 2nd–3rd day.	Died with gross kidney lesions and marked oedema.
24	4½	46	4·6	1	Appeared well throughout day.	Died during night. Marked liver and kidney damage.
42	4½	32	2·9	5	Diabetic. Apathetic. No food eaten for 4 days and anuria for last 2 days.	Killed when in condition of collapse. Marked liver and kidney damage.
6	6	41	3·7	9	Diabetic. Apathetic after about 4 days and in condition of collapse by 9th day.	Ditto.
9	6	48	4·3	9	Ditto.	Ditto.
258	2	38	3·4	8	Ditto.	Ditto.

Animal No. 24 died within 24 hours. No abnormality was detected at autopsy except the pink nature of the subcutaneous and omental fat. Usually an intense hyperaemia of the skin vessels, particularly along the back, and extensive subcutaneous haemorrhage, either petechial or diffuse, were observed; the latter lesion was distributed particularly in the tissue over the vertebral column extending from T8–L6. This occurred most noticeably in No. 42 and was evident in Nos. 6, 9 and 258. It was quite prominent in the two animals (Nos. 119 and 623) killed 13 hours after injection, and was the only gross abnormality noted in these cases. Extensive oedema was observed in three animals (Nos. 861, 42 and 258). This was particularly pronounced in No. 861 in which the peritoneal cavity was awash with fluid countaining blood and loose fibrin clots. Extensive hyperaemia of the vessels of the alimentary tract was also observed in these animals. Another general feature revealed at autopsy of most of the animals was the pink colouration of the subcutaneous and omental fat and the decidedly pink colour assumed by a cut surface of the kidney when exposed to the air for a few minutes. This colour is, no doubt, due to the persistence of reduction products of alloxan (Archibald, 1945) in the tissues.

Histological examination revealed that alloxan may bring about acute degenerative changes in the kidney. The extent of this damage was sufficient to indict it as the main cause of the serious decline and of the death of some of the cases.

In the affected kidneys the glomeruli, apart from some congestion, were generally normal. The cytoplasm of the cells of the convoluted tubules was granular and vacuolated. The cells lining the third convoluted tubule were desquamated and the lumen frequently occluded with casts. Numerous vacuoli were evident in the cytoplasm of the cells of Henle's loop. This kidney damage occurred particularly in animals Nos. 258, 861 and 24 and to a lesser extent in Nos. 6, 9 and 42. The kidneys of the remainder of the animals were either normal or had only minor lesions. Casts were evident in the collecting tubules and some granular and reticular

material in the convoluted tubules of the kidney of animal No. 203, but no extensive damage was observed. Similar but even less noticeable changes were observed in the kidney of No. 637. Sections of the liver and adrenal from every animal were not examined but the general picture indicated that here again in those animals which collapsed and died there was sometimes extensive tissue breakdown. The liver on these occasions showed evidence of a severe fatty infiltration. There was a marked venous congestion of the adrenal medulla in animal No. 258. No abnormality was detected in these tissues from animals Nos. 203, 637, 119 and 623.

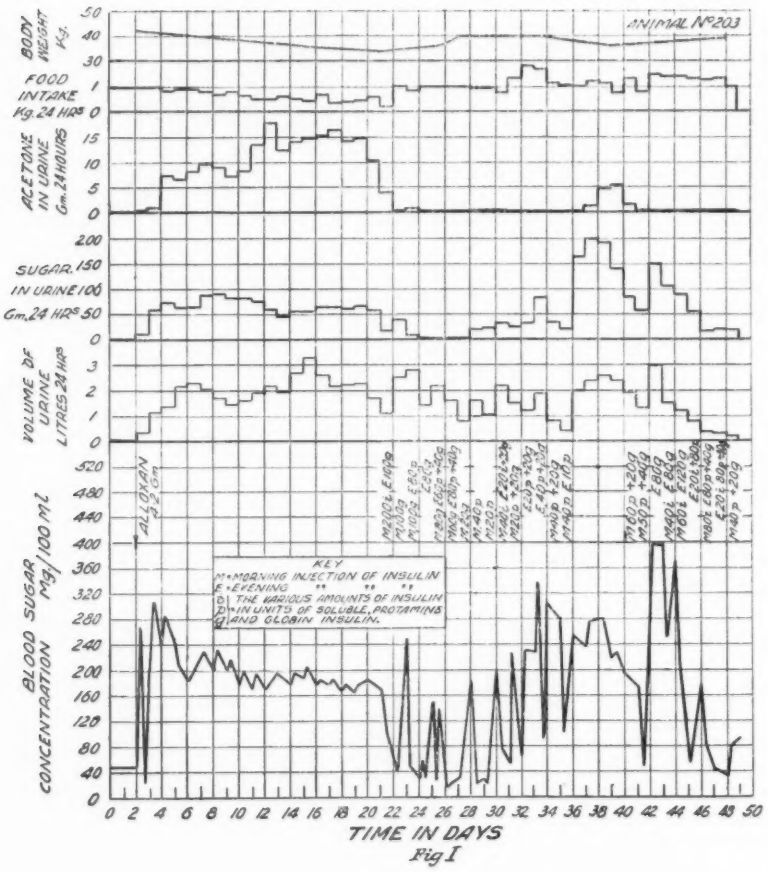

ANIMAL Nº 203
BODY WEIGHT Kg.
FOOD INTAKE Kg. 24 HRS
ACETONE IN URINE Gm. 24 HOURS
SUGAR IN URINE Gm. 24 HRS
VOLUME OF URINE LITRES 24 HRS
BLOOD SUGAR CONCENTRATION Mg/100 ml
ALLOXAN 0.2 Gm
KEY
M = MORNING INJECTION OF INSULIN
E = EVENING " "
(B) THE VARIOUS AMOUNTS OF INSULIN
D = IN UNITS OF SOLUBLE, PROTAMINS AND GLOBIN INSULIN.
TIME IN DAYS

Fig I

The histology of the pancreas was more fully observed. Differential staining of α and β cells of the islet tissue was attempted but so far this has not been sufficiently clear to demonstrate the two types of cells in the sheep. The method described by Gomori (1941) was followed meticulously. Although in preliminary tests it proved entirely successful for differentiating these cells in the pancreatic tissues of the rabbit, guinea-pig and the ox, on no occasion was it possible here to produce a clear differentiation of the islet tissue of sheep's pancreas. The distribution of the islets in the pancreas of the sheep is highly variable. The islets usually are small and consist of only a few closely packed cells. Frequently the cytoplasm is indistinct and when stained by Gomori's method it usually takes on a diffuse muddy pink colour throughout. In view of this it has not been possible to draw any conclusions as to the specific necrosis of the β cells which has been observed by other workers with non-ruminants. However, some signs of degenera-

tion in both acinar and islet tissue were evident in most of the sections. Those from two animals killed 13 hours after the injection showed marked degeneration of islet tissue and some swelling and droplet formation in the cytoplasm of the acinar cells. The nuclei of the islet cells were observed to be in all stages of degeneration and the cells appeared to have receded from the basement membrane. In the sections from No. 119 there was a suggestion of two types of cells; some of those which took on a more bluish hue were markedly degenerate and there was evident pyknosis in some nuclei of those which stained pink.

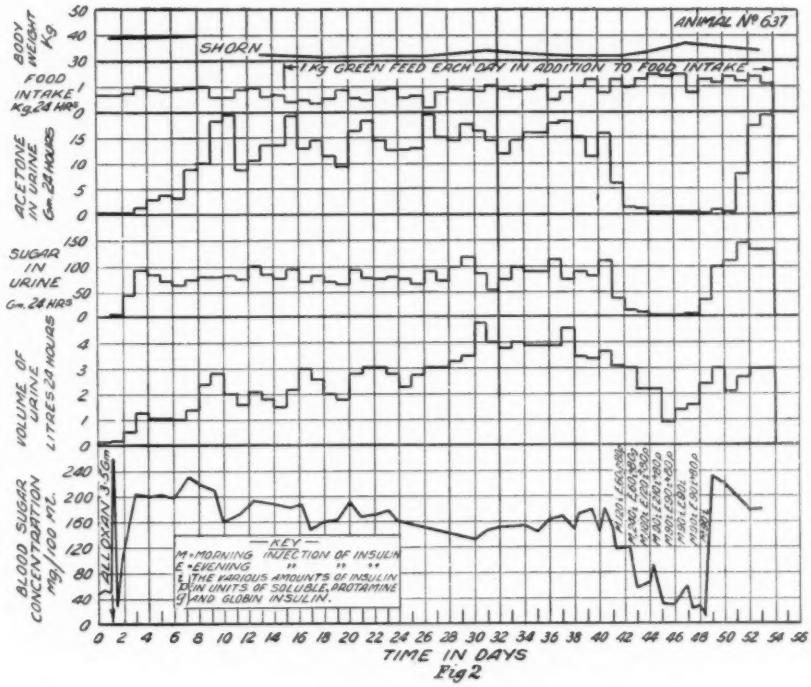
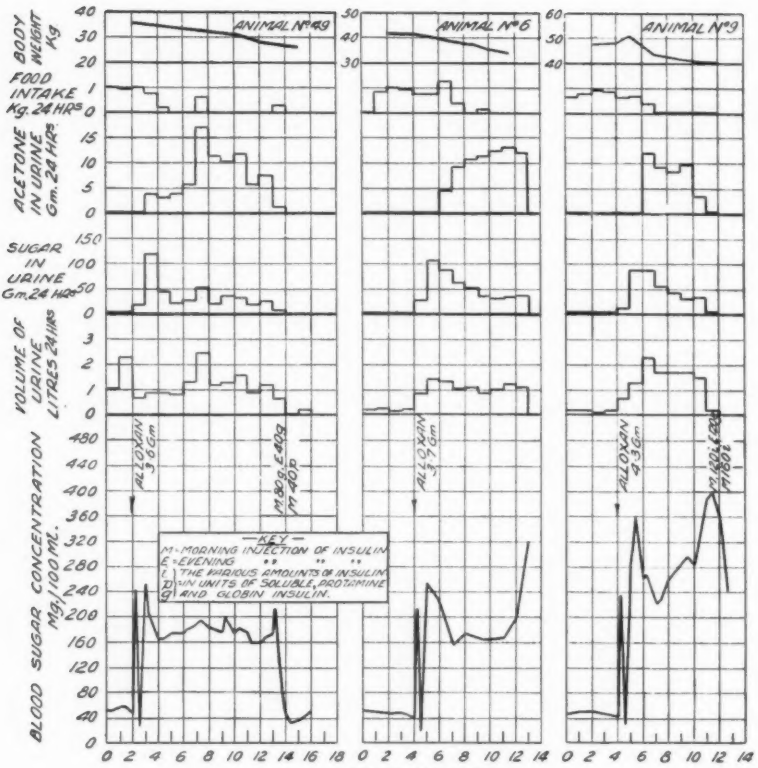

ANIMAL Nº 637
SHORN
1 Kg GREEN FEED EACH DAY IN ADDITION TO FOOD INTAKE
BODY WEIGHT Kg
FOOD INTAKE Kg 24 HRS
ACETONE IN URINE Gm 24 HOURS
SUGAR IN URINE Gm 24 HRS
VOLUME OF URINE LITRES 24 HOURS
BLOOD SUGAR CONCENTRATION mg/100 ML.
ALLOXAN 3·5 Gm
KEY
M = MORNING INJECTION OF INSULIN
E = EVENING " " "
THE VARIOUS AMOUNTS OF INSULIN IN UNITS OF SOLUBLE, PROTAMINE AND GLOBIN INSULIN.
TIME IN DAYS

Fig 2

In the sections from those animals which were killed when in an acute condition, Nos. 6, 9 and 258, obvious changes were observed and the pancreas of animal No. 49 was markedly affected. Hydropic degeneration was one of the main features: the islets of No. 49 indicated the terminal stages of this. Here the outline of the islet cells was indefinite and the cytoplasm in many cases was drawn up into droplets. Some nuclei were enlarged, but many had disappeared altogether. The changes in the acinar tissue suggested an abnormally increased secretory activity; each cell showed signs of enlargement and the ducts were dilated. The acinar tissue from the two animals which were maintained in a diabetic condition for 47 and 52 days respectively was normal, and extensive damage was not apparent in the islet tissue. Some of the islet cells, however, did show signs of nuclear degeneration and of changes in the cytoplasm. It was not possible, however, to determine whether the surviving cells were of the α or β type.

Attempts to determine the insulin content of the pancreas were unsatisfactory. In one analysis of the pancreatic tissue taken from a normal sheep some success was obtained by using the mouse method with 60 mice in two groups of 30. This assay yielded an estimate of approximately 0·7 units of insulin per gm. of pancreas, but on other occasions the number of mice available was too few to give results which were sound statistically. The figure, 0·7 units of insulin per gm. of sheep's pancreas, agrees reasonably with that quoted by Haist (1944). With the rabbit method, however, the results were most erratic; the material extracted for assay did not bring about the expected reduction of the blood sugar concentration. On some occasions injection of the extracts produced a rise in the blood sugar, similar to that which frequently follows injection of similar extracts prepared from other tissues.


BODY WEIGHT Kg
FOOD INTAKE Kg. 24 HRS
ACETONE IN URINE Gm. 24 HRS
SUGAR IN URINE Gm. 24 HRS
VOLUME OF URINE LITRES 24 HRS
BLOOD SUGAR CONCENTRATION Mg./100 ML.
ANIMAL No 49
ANIMAL No 6
ANIMAL No 9
ALLOXAN 3·6 Gm.
ALLOXAN 3·7 Gm.
ALLOXAN 4·3 Gm.
M 30 g. E 40 g.
M 40 D
M 60 g. E 60 g.
M 80 D
—KEY—
M = MORNING INJECTION OF INSULIN
E = EVENING " " "
( ) THE VARIOUS AMOUNTS OF INSULIN
D = IN UNITS OF SOLUBLE, PROTAMINE
G = AND GLOBIN INSULIN.
TIME IN DAYS
Fig 3
Fig 4
Fig 5

DISCUSSION.

It is evident from the above experiments that, following a single intravenous injection of alloxan, the response in the sheep is similar to that which is reported in the literature to supervene in non-ruminants. From the behaviour of the animals studied here the optimal dose of alloxan necessary for the production of a diabetic condition without extra-pancreatic complications is of the order of 90 mg. per kg. body weight. There is a marked individual idiosyncrasy, however, and the results indicate that the sheep is more prone to the toxic effects than the other animals that have been studied. While sheep respond to a single intravenous injection with the typical triphasic blood sugar reaction they apparently suffer no distress during the hypoglycaemic phase, and whereas with some non-ruminants, particularly the rabbit, it has been found advisable to administer glucose at this period, this was not necessary with sheep.

The main interest of these experiments centres around those sheep that survived the injection and developed the diabetic syndrome without other complications. The case histories of Nos. 203 and 637 show that a sheep may be maintained in a diabetic condition for at least 2 months without any marked signs of an acute deterioration in condition. In so far as hyperglycaemia, glycosuria, ketonuria, polyuria and polydipsia are part of the diabetic syndrome,

the symptoms of these animals conformed to those of diabetes mellitus. The average excretion of glucose was about 60 gm. per day, and the total ketone bodies in the urine, expressed as acetone, rose from a normal average value of 0.13 gm. to about 12 gm. per 24 hours.

Fasting for up to 7 days did not appreciably alter the urinary sugar or urinary acetone excretion of sheep with alloxan diabetes. Apparently, then, the diabetes in these sheep was not of the "mild" type observed by Lukens in partially depancreatectomised goats which, when fasted, passed only small amounts of sugar and little or no ketone bodies. The response of animals Nos. 637 and 203 indicated that the diabetic syndrome may be controlled by insulin injections. Apparently, however, sheep with alloxan diabetes are more sensitive to insulin than normal sheep. The blood sugar of No. 203 fluctuated markedly while the animal was under insulin therapy. With No. 637, however, a better control was obtained by subcutaneous injection of about 90 units of soluble insulin in the morning and 90 units of soluble and 80 units of protamine insulin in the evening. Both of these animals showed signs of hypoglycaemic coma, animal No. 203 on the evening of the 26th day and on the afternoon of the 29th day, and animal No. 637 on the afternoon of the 48th day. The former animal recovered spontaneously. Intravenous injection of 20 gm. glucose brought about immediate recovery in the latter. The blood sugar concentration just prior to this injection was reduced to about 8 mg. per 100 ml.

Thus, while it has been established that a diabetic condition can be produced in the sheep by a single intravenous injection of alloxan, several interesting points are raised. Evidence obtained from studies of the mechanism of alloxan diabetes (Goldner and Gomori, 1944; Hughes, Ware and Young, 1944; and Ridout, Ham and Wrenshall, 1944) suggests that alloxan exerts its diabetogenic effect by direct action on the pancreas. The initial hyperglycaemia is considered to be due to adreno-sympathetic stimulation, the following hypoglycaemia to the action of insulin liberated from degenerating islet cells and the subsequent permanent hyperglycaemia and diabetes due to lack of insulin. The degenerative process seems to be confined mainly to the β cells of the islet tissue and results of extensive histological examinations of the changes occuring in the islet tissues have been reported (Goldner and Gomori, 1943b; Hughes, Ware and Young, 1944; Shaw Dunn, Duffy, Gilmour, Kirkpatrick and McLetchie, 1944; and Shaw Dunn and McLetchie, 1943). Houssay, Orias and Sara (1945) have suggested an extra-pancreatic effect during the hypoglycaemic phase and have stressed the importance of the liver during the three phases of the blood sugar reaction.

The sheep conformed to the same general response exhibited by other animals that do not ruminate and it is suggested that the same set of reactions may follow the injection of alloxan. No hypoglycaemic distress was evident in the second phase of the immediate blood sugar response and this agrees with the experience that it is necessary to maintain a very low level for some time in the sheep before any symptoms become evident. If the concentration of insulin in the pancreas of sheep which is reported by Haist (1944) and suggested in this work, is accepted, then an average of only about 30 units of insulin would be liberated from the degenerating islet tissue. This amount of insulin would be insufficient to lower the concentration of sugar in the blood to the required level and maintain it there for the length of time necessary to produce the apathy and subsequent coma characteristic of extreme hypoglycaemia in the sheep. Although no specific necrosis of the β cells of the islet tissue could be demonstrated in these animals the sustained hyperglycaemia and related effects would indicate that the mechanism for the production of insulin had suffered serious damage and that this damage is associated with degenerative changes in the pancreas where both islet and acinar tissues are affected.

The intermediary metabolism of sheep may proceed through channels that differ from animals that do not ruminate, but there is no doubt that the disordered metabolism typical of alloxan diabetes can be produced in a ruminant. The diabetic ruminant will be a particularly useful accessory aid in the study of intermediary carbohydrate and fat metabolism.

SUMMARY.

A sustained diabetic condition in the sheep following a single intravenous injection of alloxan has been produced.

There is a typical triphasic response of the blood sugar concentration. The sustained hyperglycaemia is accompanied by the marked glycosuria and ketonuria of diabetes mellitus. The diabetic syndrome could be controlled with insulin injections.

The dose of alloxan necessary for the production of an uncomplicated diabetic condition is of the order of 90 mg. per kg. body weight.

There appears to be marked individual idiosyncrasy, the sheep being particularly susceptible to gross kidney and liver damage.

Changes in both the acinar and islet tissue of the pancreas followed injection of alloxan, and owing to the difficulty of differentiating between the α and β cells in the sheep it was not possible to prove that the syndrome depended entirely on specific necrosis of the β cells.

Acknowledgments. The author is indebted to Dr. J. B. Thiersch of the Institute of Medical and Veterinary Science for helpful discussion of the histopathological findings. The interest and help of colleagues at the Nutrition Laboratory was greatly appreciated and particular acknowledgment is made to Mr. H. R. Marston, Chief of the Division, for his constructive advice throughout and for his criticism and help in the preparation of the manuscript.

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A HERPES SKIN TEST REAGENT FROM AMNIOTIC FLUID

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(Accepted for publication 10th April, 1946.)

In a previous publication (Nagler, 1944) it was shown that a specific cutaneous reaction can be elicited in herpetic individuals by intracutaneous injection of heated herpes simplex virus prepared from infected chorioallantoic membranes. The present communication dscribes a more specific herpes skin test reagent obtained from the amniotic fluid of infected chick embryos and briefly reports some unsuccessful attempts at immunization with this antigen in human beings.

GROWTH OF HERPES VIRUS IN THE AMNIOTIC CAVITY OF CHICK EMBRYOS.

The strain of herpes simplex (W) was originally isolated from a typical lesion on the lip of a person, subject to recurrent labial herpes, by inoculation of vesicle fluid on the chorioallantois. It had undergone twenty passages on the chorioallantois before being used in the present work.

Anderson (1940) reported that herpes simplex virus can multiply in the wall of the amniotic sac after inoculation into the amniotic cavity of 13-day embryos. Beveridge (1944) found that high titre fluid of low protein content could be produced by inoculation of the amniotic cavity of 8-day embryos. After two days' incubation the virus in the amniotic fluid reached a titre of 10^7–10^8 per ml.

In the course of the present studies, it was observed that after injection of herpes virus into the yolk sac of 8-day embryos the amniotic fluid developed a high virus content. This observation was made before Levens and Enders' (1945) paper on a similar finding with mumps virus came to our notice. The phenomenon with mumps virus has been fully confirmed by Beveridge, Lind and Anderson (1946).

Titres obtained in the amniotic fluids three days after yolk sac injection had, in a number of experiments, an average of 1×10^7–8×10^7 per ml. The volume of amniotic fluid recovered after yolk sac inoculation was usually 2 ml. compared with 1 ml. after direct amniotic injection. In one experiment, for instance, 20 eggs after yolk sac inoculation yielded an average of 2·1 ml. amniotic fluid, whereas 16 eggs inoculated directly into the amniotic sac gave an average of 0·9 ml.

COMPARISON OF AMNIOTIC AND CHORIOALLANTOIC MATERIAL IN SKIN TEST METHODS.

Materials and Methods.

Chick embryos, 8 days old, were inoculated into the yolk sac with virus, grown either on the chorioallantois or in the amniotic cavity. The eggs were incubated at 35° C. for another three days when the amniotic fluids were removed and tested for virus content by titration on the chorioallantois. Only those fluids containing at least 10^7 living virus particles per ml. were used as skin test reagents. They were heated to 65° C. and the usual tests for bacteriological sterility and absence of active virus were made. The same batch (A III) was used throughout. Control materials were obtained from normal amniotic fluids and treated in the same way as the viral reagent. The protein content of the fluids was estimated by a micro-Kjeldahl method and a suitable dilution was made to bring the nitrogen content of the reagent and the control to the same level. The protein content of normal amniotic fluid of 11-day embryos was found to be approximately 0·005 p.c., and in infected living embryos 0·012 p.c. The protein content of infected amniotic fluids taken from embryos which had just died was much higher, approximately 0·1 p.c.

Skin test reagent (C IX) and control material were also prepared from the chorioallantois as described in the previous paper. Before heating to 65° C. the virus content of this reagent was adjusted to that of the amniotic reagent by suitable dilution with saline.

A third antigen (PA VII) was prepared by my colleague, J. F. McCrea, from amniotic fluids by adsorption on to freshly precipitated calcium phosphate. It was primarily designed for immunization experiments in rabbits but was also tested for its suitability as a skin test reagent. For his purpose its virus content was adjusted to that of the two other reagents described. The method of precipitation and adsorption will be described in detail elsewhere (McCrea unpublished).

Skin tests were made by intracutaneous injection of 0·1 ml. reagent, diluted to 1 in 50, into the skin of the flexor surface of the forearm. The diameter of the erythema resulting was measured at 24 and 48 hours. All of the subjects inoculated were members of the laboratory staff. They were classified as herpetic or non-herpetic according to their clinical history and the serum neutralization test on the chorioallantois using the method of Burnet and Lush (1939). In all instances except one, mentioned later, the neutralization test agreed with the clinical history.

Results.

The results of the cutaneous tests with the amniotic reagent A III in adults subject to labial herpes are given in Table 1. It will be noticed that all gave positive reactions to the A III preparation whereas only in one instance did the normal amniotic fluid control give even a trivial nonspecific reaction. This contrasts with the relatively high proportion of nonspecific reactions observed both in our previous and our present work with control preparations from normal chorioallantoic membranes. The difference is apparently due to the very low protein content of the amniotic fluid.

TABLE 1.

Tests for cutaneous reactivity to herpes reagent A III in persons subject to herpes.

	Erythema in mm.			
	Herpes antigen.		Control.	
Subjects.	24 hr.	48 hr.	24 hr.	48 hr.
G.A.	7	4	0	0
N. McA.	10	11	0	0
J. McC.	11	10	0	0
M.D.	11	10	0	0
B.E.	10	11	0	0
M.K.	9	5	0	0
J. McL.	5	4	0	0
R.L.	8	0	0	0
D.N.	14	4	0	0
M. McP.	14	12	0	0
N. McR.	11	0	0	0
J. St.	11	0	0	0
J.T.	12	10	0	0
F.W.	7	0	0	0
A.W.	9	10	2	0

One subject, not included in the above group, gave a skin reaction of 5 mm. diameter with the herpes reagent A III, and no reaction with normal fluid. The test was repeated with the same result. A sample of this subject's serum taken six months previously was still available and was shown to be free of antibody. A sample taken at the time of the first skin test gave a slight inhibition in the neutralization test and a sample taken six weeks afterwards showed typical neutralization. There was no history of any recurrent herpes and no indication of any possible primary lesion except for a staphylococcal infection on the leg a few weeks before the skin test was done. This is the second case in the course of our investigations in which a primary herpetic infection in an adult could be shown.

Fifteen non-herpetic individuals were also injected with reagent A III. All persons in the group showed no reaction either to the herpes reagent or to the normal amniotic fluid control.

Table 2 shows the result of a comparative test with the amniotic reagent A III and the chorioallantoic reagent C IX in three individuals. Both reagents had the same virus content, approximately 2×10^7 per ml.—they were injected into corresponding sites of both forearms in dilutions of 1: 50, 1: 100 and 1: 250. No significant difference in the reactivity of these two reagents could be found and the amount of virus inoculated was apparently the only factor responsible for the size of the erythema. Control injections with normal amniotic fluid and membranes gave no reaction in these three subjects.

TABLE 2.

Comparative herpes skin tests, using amniotic reagent A III and chorioallantoic reagent C IX.

	Erythema in mm.					
	Reagent A III.			Reagent C IX.		
Subjects.	1/50	1/100	1/250	1/50	1/100	1/250
N. McA.	8	6	3	9	7	5
D.N.	10	6	5	9	8	4
J.T.	13	9	4	13	6	2

The calcium phosphate reagent PA VII was injected into six herpetic and four non-herpetic individuals. The skin reactions in the herpetic subjects were smaller than those obtained with A III but lasted longer, usually forming a small firm nodule, which persisted for more than a week. Three of the non-herpetic subjects gave similar reactions. Apparently the reactions were due largely to the calcium phosphate and, therefore, this material is unsuitable for use as a skin test reagent.

ATTEMPTS AT ACTIVE IMMUNIZATION ON HERPETIC INDIVIDUALS.

Six herpetic subjects were injected subcutaneously with formolized (0·08 p.c. of HCHO) amniotic fluid containing 2×10^7 virus particles per ml. Three inoculations of 0·5, 1·0 and 1·0 ml. respectively were made at weekly intervals. The subjects were kept under observation for six months to determine whether the occurrence of herpetic lesions had been prevented. Four of them did experience fresh herpetic eruptions on their lips in association with attacks of common cold. It was concluded that these injections had no beneficial effect.

SUMMARY.

A herpes simplex skin test reagent, prepared from infected amniotic fluids has been tested in a number of herpetic and non-herpetic individuals and has given more specific results than a chorioallantoic reagent previously used. The amniotic reagent is easier to produce and contains only a trace of nonspecific proteins.

Attempts at immunization with a formolized amniotic reagent were unsuccessful.

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ON SOME PROPERTIES OF CRUORATIN AND ITS RELATIONSHIP TO PSEUDO-HAEMATIN

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(Accepted for publication 29th March, 1946.)

Of the various products of different oxidative disintegrations of the blood pigment, e.g. sulphaemoglobin, "pseudo-haemoglobin" (Barkan and Schales, 1937, 1938) "choleglobin" (Lemberg, Legge and Lockwood, 1939) etc., the prosthetic group of one only, cruoratin (Holden, 1945) has been detached from the accompanying protein as an iron-containing pigment. The porphyrin of sulphaemoglobin (Haurowitz, 1941) and the iron-free bile pigment of choleglobin (Lemberg, Legge and Lockwood, 1941) are reported to have been isolated. Cruoratin is, unfortunately, very unstable, but a method for the preparation of dilute solutions was outlined in a previous paper, (Holden, 1945). The present communication records further studies in its preparation and properties and also the preparation of pseudo-haematin, apparently a closely allied pigment.

PREPARATION OF CRUORATIN.

It was found, especially when it was desired to dispense with the use of ammonia compounds in the preparation, that the acidity of the sodium bisulphite produced by the oxidation of the sodium hyposulphite materially retarded the formation of cruoralbin and that this proceeded more favourably in a solution of about pH 9·5–10·5. Investigation showed that at low temperatures cyan-methaemoglobin can withstand a high concentration of potassium cyanide without being denatured and so unfitted for the production of cruoralbin. At a little above 0° C. in the presence of much cyanide the spectrum of cyan-haemochromogen appeared on reduction with sodium hyposulphite without the protein being (irreversibly) denatured. This cyan-haemochromogen does not appear to form cruoralbin on oxidation but, on its solution being made less alkaline, reverts to the compound with the peculiar two-banded spectrum which is obtained on the reduction of cyan-methaemoglobin within the range pH 7–10. Concerning its constitution there is not yet agreement but it appears to be an intermediate in the production of cruoralbin.

The yield of cruoratin is, at the best, poor; much of the pigment remains inseparable from the protein or undergoes some decomposition during the process of extraction. The following procedure gives the best yield obtained from a day's work. Fifty c.c. of horse erythrocytes were mixed in a litre bottle at 18–25° C. with 25 c.c. 10 p.c. potassium cyanide solution and left for a few hours till the α-band of oxyhaemoglobin was nearly invisible and most of the pigment converted to cyan-methaemoglobin. The bottle was placed in a cold room at 0° C. till the next day. A further 75 c.c. of potassium cyanide were added and the bottle rotated horizontally in an ice chest while a slow current of oxygen was passed through. Small amounts of sodium hyposulphite were added and the spectrum watched. At the first reduction it was that of "cyan-haemochromogen" but soon changed to that of cyan-methaemoglobin and, on the continued addition of sodium hyposulphite, to that of "reduced cyan-methaemoglobin" (a two-banded spectrum greatly resembling that of denatured globin haemochromogen). When this stage was reached the reducing agent subsequently added until the end of the reaction consisted of a mixture of equal weights of sodium hyposulphite and anhydrous sodium carbonate. This kept the solution sufficiently alkaline for the formation of cruoralbin to proceed at its best speed. When nearly all the proto-haematin derivatives had apparently been decomposed, the bottle was placed in the cold room at 0° C. The pigment was extracted with amyl alcohol and acetic acid as already described. As much acid as possible was washed from the alcohol layer. Normal sodium hydroxide was added with shaking till an examination of the liquid with a pocket lens showed that the pigment had passed to the aqueous phase. The mixture was centrifuged, the amyl alcohol layer discarded to residues and that dissolved in the water removed by distillation under a vacuum of a few mm. For some purposes, when the presence of ammonia was allowable, concentrated ammonia was used instead of sodium hydroxide.

[1] This work was aided by a grant from the National Health and Medical Research Council.

Solutions of cruoratin cannot be kept satisfactorily even at 0° C. Some in an evacuated flask lasted better than specimens kept in corked flasks exposed to the air. A small amount dried and stored over solid sodium hydroxide in a thoroughly evacuated desiccator at 0° C. has proved the most stable, but after several months it too shows signs of decomposition.

CHROMATOGRAPHY.

Many experiments were made in an attempt to utilize chromatographic methods in the isolation and purification of cruoratin. The results, however, are so meagre that a brief summary only is justified. Cruoratin is free from bile pigments which is fortunate since its instability in acid solutions prevents the usual removal of bile pigments with hydrochloric acid. It is contaminated by a good deal of proto-haematin and by tawny indeterminate breakdown products possibly resulting from the extraction process. After many trials with a wide variety of natural adsorbents, such as clays and other minerals and synthetic adsorbents ranging from samples of alumina to resins and other organic chemicals, the only class of substance that offered the smallest prospect of success appeared to be some of the insoluble inorganic salts. A number were prepared usually by running a dilute solution of a soluble salt of the metal into an excess of a dilute solution of a soluble salt of the acid radicle. This was generally done at 60–80° C., though some were prepared at about 20° C. The quantities were so chosen that only about 2–3 gm. of the salt resulted from each litre of the united volumes of the reactants. Preparations made from stronger solutions appeared to have a diminished specificity. The effect of the temperature on the character of the salt for this special purpose did not appear to be great. The aqueous solutions of cruoratin were chromatographed at 0° C. and pH 8–9. From more alkaline solutions the haematin was not absorbed. When it had been adsorbed it was readily eluted by such alkaline fluids. A few experiments at pH 6 showed that at this pH also haematin was not readily adsorbed from solutions containing it and cruoratin. Organic solvents eluted adsorbed haematin and so were useless.

The results of the chromatographic experiments are shown in Table 1. The majority of the adsorbents effected little separation of the haematin from the cruoratin. A troublesome feature was that a compound that absorbed the former admirably when alone had a greatly diminished affinity for it in the presence of the latter. Little success was achieved in the removal of the tawny decomposition products. The degree of separation was assessed with the aid of an old König-Marten spectrophotometer calibrated against a set of arbitrary units. Barium oxalate was the best of the adsorbents tried and it was further somewhat improved by using an excess of barium chloride in its preparation. A variation of the technique was to precipitate with baryta solution most of the haematin as barium haematinate and then to filter the solution first through a paper and then through a much smaller column of barium oxalate. The results were similar to those otherwise obtained.

TABLE 1.

The chromatographic behaviour of cruoratin and proto-haematin.

C = cruoratin; H = protohaematin; 1 = strongly adsorbed at the head of the column; 2 = adsorbed throughout the column; 3 = little adsorbed; B = column blocks the passage of water too much to be of use.

Adsorbent.	Behaviour.	Adsorbent.	Behaviour.
$MgCO_3$	H_1, C_2	CaC_2O_4	H_1, C_2, B
$CaCO_3$	H_1, C_1	SrC_2O_4	H_2, C_3
$SrCO_3$	H_1, C_1	BaC_2O_4	H_2, C_3
$BaCO_3$	H_1, C_2	ZnC_2O_4	H_1, C_1
$PbCO_3$	B	$FePO_4$	H_1, C_1
$PbCO_3$ on silica	H_2, C_2	$Ba_3(PO_4)_2$	H_1, C_1, B
$SrSO_4$	H_2, C_3, B	Silica	H_3, C_3
$BaSO_4$	H_1, C_1, B	Clays	H_1, C_1
$PbSO_4$	H_1, C_1, B	Synthetic Resin I.R.4	H_2, C_2
PbO	H_1, C_1		

Whenever cruoratin was adsorbed on a column efforts to elute it were invariably unsuccessful and yielded mostly break-down products.

ANALYSES.

The fact that, unlike choleglobin, cruoratin is not readily converted into bile pigments, led to the determination of the ratio of iron to nitrogen in solutions of cruoratin to see if any light would be thereby thrown on the difference between it and choleglobin. Three of the best solu-

tions of cruoratin which had been prepared without the use of any nitrogenous reagent except the potassium cyanide were analysed for iron by the di-pyridyl and for nitrogen by the micro-Kjeldahl method. As can be seen from Table 2, there were found about eight atoms of nitrogen for each iron atom. Thus there is apparently no question of any deficiency of nitrogen. It would appear likely that about half of that found is from contaminating substances.

TABLE 2.

Substance.	Iron. mg. per c.c.	Nitrogen. mg. per c.c.
Cruoratin	0·106	0·220
Cruoratin	0·043	0·078
Cruoratin	0·086	0·174
Pseudo-haematin	0·071	0·126

PSEUDO-HAEMOGLOBIN AND CRUORATIN.

While it seems impossible at present to indicate the relationship, if any, between cruoratin and some of the green pigments derived from haemoglobin that between cruoratin and the pseudo-haemoglobin of Barkan and Schales may be less obscure. The preparation of pseudo-haemoglobin has been modified as follows: 50 c.c. of horse red cells laked with 50 c.c. of water and 50 c.c. of 10 p.c. potassium cyanide are separately cooled overnight to 0° C. Next morning the cyanide is added to the laked cells and the whole quickly mixed. Immediately 4 c.c. of 100 vol. hydrogen peroxide diluted with an equal volume of ice-cold water are added and the mixture left in an ample jar at 0° C. When the spectrum of oxyhaemoglobin has nearly disappeared the pigment is detached from the protein at 0° C. with amyl alcohol and acetic acid. This and the subsequent steps are the same as for cruoratin. The essential features of this preparation are to use a lot of cyanide but to give it as little chance as possible of forming cyan-methaemoglobin before the hydrogen peroxide can effect the decomposition to pseudo-haemoglobin. The low temperature also greatly facilitates the separation from the protein of the prosthetic group.

The pigment so obtained yields a CO derivative whose absorption band is similar to that of CO cruoratin. It behaves similarly on a barium oxalate column. It has much less haematin to be removed but on the other hand a small amount of biliviolin can be extracted using ether and hydrochloric acid extractions.

The reports of the various cyano-compounds of pseudo-haemoglobin and its derivatives would appear to rest on rather frail spectroscopic evidence derived from grossly contaminated material. No really trustworthy evidence shows the existence of a cyanide compound of either oxidized or reduced cruoratin or pseudo-haematin.

TABLE 3.

Comparative spectrometric observations on derivatives of cruoratin and of pseudo-haematin.

A. Determinations made with a Hartridge spectrometer.

Substance.	λ, mμ.	Substance.	λ, mμ.
Cruoraem	608	Pseudo-haem	619
Cruoraem with KCN	608	pseudo-haem with KCN	619
Cruoraem CO	615	pseudo-haem CO	624
Cruoraem CO denat. horse globin	620·5	pseudo-haem CO denat. horse globin	631

B. Determinations made with a photo-electric spectrophotometer.

Substance.	λ, mμ.	$\epsilon 10^{-3}$.
Cruoraem CO	614	20
pseudo-haem CO	623	22

C. Ultra-violet bands.

Substance.	λ, mμ.	$\epsilon 10^{-3}$.
Cruoraem CO	408	70
pseudo-haem CO	410	50

The value for cruoraem CO given in the previous paper was unfortunately due to an error of calculation.

The values in section A were determined on solutions that contained no ammonia.

There is no *a priori* ground for thinking that the biliviolin is formed from the pseudo-haematin either by the amyl alcohol and acetic acid or by the hydrochloric acid used to extract it. There seems no valid reason for rejecting the hypothesis that the bile pigment is formed by a side reaction involving hydrogen peroxide and that its absence from cruoratin solutions is due to the absence of sufficient hydrogen peroxide for its formation. One is then left with the problem of the nature of the reactions between oxygen and "reduced cyan-methaemoglobin" on the one hand and hydrogen peroxide and oxyhaemoglobin (or perhaps haemoglobin) in the presence of cyanide on the other which seem to lead to closely allied pigments. The fact that the oxygen and the hydrogen peroxide cannot exchange tasks is opposed to the common opinion that the immediate product of the reduction of cyan-methaemoglobin is a "cyan-haemoglobin" analogous to, say, carbon monoxide haemoglobin.

SUMMARY.

Chromatographic experiments on the preparation of cruoratin showed that barium oxalate is helpful in separating it from proto-haematin. Determinations of the iron/nitrogen ratio on solutions of cruoratin throw no light on the inability to convert cruoratin into bile pigments. A pigment closely allied to cruoratin but mixed with small amounts of bile pigments has been extracted from specimens of "pseudo-haemoglobin" prepared at 0° C.

Acknowledgments. The author is indebted to Mr. A. T. Dick of the Council for Scientific and Industrial Research for the determination of a visible absorption curve.

The ultra-violet spectrophotometer is the property of the Australian National Health and Medical Research Council.

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THE EFFECTS OF MINERAL NUTRIENTS ON THE CONCENTRATION OF ASCORBIC ACID IN LEGUMES AND TWO LEAF VEGETABLES

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(Accepted for publication 5th March, 1946.)

The heavy metals, copper, zinc, manganese, iron and molybdenum are now accepted as essential plant nutrients, and their probable significance as key factors in enzyme systems has suggested that they may directly or indirectly influence vitamin synthesis.

The development of technical reliability in the determination of ascorbic acid in plant material, Lugg (1942) and in the location of micronutrient and potash deficiency by the response of herbage legumes in controlled soil cultures, Ferres and Trumble (1943), made possible the work herein described.

The literature concerning variation of ascorbic acid content with applications of micronutrients has been reviewed by Maynard and Beeson (1943), who found it too contradictory to give any definite conclusion. They quoted two instances of increase in ascorbic acid with zinc in oranges, Roy and Bahrt (1940) and Cowart (1941), but cited evidence brought forward by Lyon, Beeson and Ellis (1943) which showed that marked increases in the growth of tomatoes following the addition of zinc were unaccompanied by any change in the concentration of ascorbic acid.

Hester and Kohman (1940) and Sugawara (1941) secured an increase in ascorbic acid with the application of potash to tomatoes and potatoes respectively. Hamner, Lyon and Hamner (1942) on the other hand, found no substantial effect of potash on tomatoes when other elements were kept sufficiently high.

Rudra (1944) has reported that "manganese has a specific rôle in the synthesis of ascorbic acid by plants and animals", and Hester (1941) has found a lower level in tomato plants untreated with manganese than those to which manganese was added. On the other hand Lyon and Beeson (1943) and Lyon, Beeson and Ellis (1943), obtained no change in the concentration of ascorbic acid with the addition of this element.

There is considerable evidence to indicate that climatic factors affect the vitamin content of plants grown for food; in particular the dominant rôle of light in the ascorbic acid content of plants has been recognized, Maynard and Beeson (1943). Work by Reid (1942) indicated that both high light intensity and long days were conducive to higher ascorbic acid values in cow peas; a sufficient supply of available major nutrients, with special reference to nitrogen, was stated to favour a higher content of ascorbic acid, provided light was available for the active synthesis of carbohydrate.

While it is well known that the ascorbic acid content of plants varies with the locality, it has been generally impossible to ascertain the specific factors involved in the environmental complex. In all cases there has been insufficient control of the individual variables concerned. No work appears to have been carried out on the possible interactions of micronutrients and climatic factors as affecting the ascorbic acid content.

Recently Hamner (1945), in a further review, concluded that variations in the ascorbic acid content of plants are so influenced by varietal differences and by climatic conditions that soil factors and the application of fertilizers are likely to have little practical importance, and that treatments which produce the highest crop yields per acre are likely also to produce the highest vitamin yield per acre.

Investigations were commenced at Adelaide early in 1944 to determine the possible relationships between yield response and the concentration of ascorbic acid developed, following the application of mineral nutrients to several legumes, silver beet and lettuce, grown on soils known to be deficient in the particular elements. A range of seedings was made to cover varying seasonal effects.

EXPERIMENTAL.

Lettuce, silver beet, subterranean clover, lucerne, strawberry clover, barrel medic and peas were grown in soils which had previously been found to be deficient in one or more of the elements K, Cu, Zn, Mn, B and Mo. The tests were conducted in small earthenware pots using the technique described by Ferres and Trumble (1943).

The experiments were planned to test the effect of single elements on both the total yield and the concentration of ascorbic acid in the plant; but, as the effect of one element was likely to be influenced by the presence or absence of others, a factorial design was used in nearly all cases. When harvested, the plants were cut immediately above the soil, weighed at once and two samples taken. The moisture content was determined from one; on the other the estimation of the concentration of ascorbic acid was begun within one hour of harvesting.

Method of Ascorbic Acid Estimation.

The ascorbic acid was extracted by pulverization of the plant material with metaphosphoric acid solution in a Waring Blendor as described by Lugg (1942). The preparation of the metaphosphoric acid followed the description by I. Brown (1942).

It was found that the relative differences in ascorbic acid contents of the samples could be estimated as accurately as desired after the formaldehyde treatment in Lugg's simplified procedure was omitted. The method of estimation was then reduced to simple titration of extract in 25 c.c. conical flasks with 2:6 dichlorphenolindophenol to a five second persistent end point. This simplification permitted the handling of a greater number of samples. The value for each sample was calculated from the average of three titrations. Titrations were carried out immediately after extraction. Dehydro-ascorbic acid was not estimated.

RESULTS OF EXPERIMENTS.

The results of all experiments are presented in the following Tables; the yields are in gm. dry matter per culture and the ascorbic acid concentration in mg. per 100 gm. dry matter.

TABLE 1.

Yield and ascorbic acid concentration of lettuce grown in a brown sand from Yalkuri, and silver beet, grown on a red brown sand from Mt. Benson, S.A.

(1) Lettuce (sown 20th April). Means of 2 cultures.

	—	Cu	Zn	K	Mo
(a) Yield (gm.).	5·74	3·56	3·94	5·27	4·64
Significant differences					2·1 (P = ·01) 1·6 (P = ·05)
(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).	40	44	45	42	44
Significant differences					10·2 (P = ·01) 7·6 (P = ·05)

(2) Silver beet (sown 16th March). Means of 3 cultures.

	—	Cu	Zn	K	CuZn	CuK	ZnK	CuZnK
(a) Yield (gm.).	1·78	1·42	1·70	1·69	1·79	1·59	1·62	1·70
Significant differences					·64 (P = ·01) ·48 (P = ·05)			
(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).	63	63	64	73	61	70	55	69
Significant differences					20 (P = ·01) 15 (P = ·05)			

The Yalkuri soil, on which lettuce was grown, had previously shown marked responses by subterranean clover to Zn and also smaller effects with K and Mo; Mt. Benson soil on which silver beet was grown, had shown an increased yield of subterranean clover following applications of Cu, Zn and K. The lettuce was harvested aften nine weeks; the silver beet was grown for eight weeks. Neither yield nor ascorbic acid concentration was influenced by the treatments.

TABLE 2.

Yield and ascorbic acid concentration of silver beet grown on a brown sand from Brinkley, and subterranean clover grown on a soil of the same type from Yalkuri, S.A.

(1) Silver beet (sown 8th April). Means of 2 cultures.

Harvest	—	Zn	K	Mn	ZnK	ZnMn	KMn	ZnKMn
(a) Yield (gm.).								
I	·31	·33	·35	·32	·35	·31	·38	·34
II	·46	·43	·38	·40	·47	·29	·43	·30
III	·48	·37	·35	·43	·41	·43	·39	·41
IV	·41	·37	·47	·52	·46	·41	·50	·48
V	·42	·54	·46	·44	·27	·44	·43	·49
Means of 10	·41	·40	·40	·42	·39	·37	·42	·40

Significant differences ·22 (P = ·01) for means of 2
·16 (P = ·05) " " " "
·10 (P = ·01) " " " 10
·07 (P = ·05) " " " "

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

I	40	44	42	43	43	47	41	49
II	43	49	44	44	41	52	44	47
III	54	53	55	60	55	57	57	67
IV	73	73	65	63	60	65	69	68
V	62	68	62	55	63	65	72	64
Means of 10	54	57	53	53	52	57	56	59

Significant differences 14 (P = ·01) for means of 2
11 (P = ·05) " " " "
6 (P = ·01) " " " 10
5 (P = ·05) " " " "

(2) Subterranean clover (sown 21st July). Means of 2 cultures.

Harvest	—	Zn	K	Mn	ZnK	ZnMn	KMn	ZnKMn
(a) Yield (gm.).								
I	·56	·65	·54	·52	·71	·65	·61	·72
II	·97	1·33	1·08	·93	1·68	1·40	1·28	1·49
III	1·07	1·51	1·51	1·08	1·88	1·68	·94	1·67
IV	1·53	2·17	1·43	1·69	2·54	2·24	1·81	2·40
V	2·74	2·91	2·74	2·79	3·29	3·28	2·28	2·98
Means of 10	1·37	1·71	1·46	1·40	2·02	1·85	1·38	1·85

Significant differences ·50 (P = ·01) for means of 2
·38 (P = ·05) " " " "
·22 (P = ·01) " " " 10
·17 (P = ·05) " " " "

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

I	86	103	75	69	104	102	76	101
II	89	102	88	91	116	104	95	102
III	85	97	82	83	92	93	79	92
IV	92	81	91	94	88	85	90	86
V	136	120	122	122	118	125	121	108
Means of 10	97	100	91	92	103	102	92	98

Significant differences 16 (P = ·01) for means of 2
12 (P = ·05) " " " "
7 (P = ·01) " " " 10
5 (P = ·05) " " " "

Silver beet was sown in Brinkley soil and subterranean clover in Yalkuri soil; the two soils are similar brown sands of the same pH which had both previously given comparable responses to Zn and K. The silver beet showed no response to the elements used either in terms of yield or ascorbic acid concentration, except that the latter was increased by Mn at the 3rd harvest ($P < \cdot 01$). On the other hand the yield of subterranean clover was increased by Zn at all harvests; and there was also a marked response to K at the first and second harvests, the levels of significance being 5 p.c. and 1 p.c. respectively for these two harvests. The concentration of ascorbic acid was not significantly affected by K, there being a tendency to depression by this element. Zn increased the concentration of the ascorbic acid of the first three harvests.

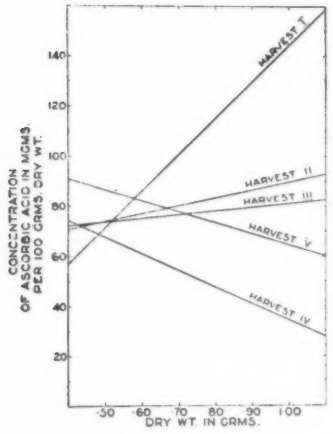

Fig. 1. Slopes of regression lines for each harvest of Table 2 (2):

Harvest I	$b = +147 \cdot 13$	$P < \cdot 001$
II	$+ 31 \cdot 19$	$< \cdot 001$
III	$+ 14 \cdot 52$	$< \cdot 001$
IV	$- 6 \cdot 71$	$< \cdot 05$
V	$- 4 \cdot 37$	—*

* Not significant.

TABLE 3.

Yield and ascorbic acid concentration of lucerne grown on a grey sand from Wanilla, and subterranean clover on a grey clay from Calperum and also on a brown loamy sand from Narrabri.[1]

(1) Lucerne (sown 21st April). Means of 3 cultures.

—	K	Mo	R*	KMo	KR	MoR	KMoR
(a) Yield (gm.).							
·39	·39	·41	·33	·54	·38	·55	·50

Significant differences ·16 (P = ·01)
·12 (P = ·05)

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

224	227	221	246	224	239	206	227

Significant differences 72 (P = ·01)
54 (P = ·05)

* R = Cu Zn B Mn.

(2) Subterranean clover (sown 23rd April). Means of 2 cultures.

—	(CuZn)	(KMnB)	Mo	(CuZn) (KMnB)	(CuZn) Mo	(KMnB) Mo	(CuZn) (KMnB) Mo
(a) Yield (gm.).							
1·75	1·95	2·01	2·28	1·88	2·49	2·34	2·54

Significant differences ·40 (P = ·01)
·30 (P = ·05)

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

110	118	110	112	109	114	114	116

Significant differences 13 (P = ·01)
10 (P = ·05)

[1] Narrabri, New South Wales. All other centres referred to are in South Australia.

(3) Subterranean clover (sown 16th May). Means of 2 cultures.

—	Zn	(KMnB)	Mo	Zn (KMnB)	Zn Mo	(KMnB) Mo	Zn (KMnB) Mo
(a) Yield (gm.).							
1·75	1·86	1·96	2·82	1·80	2·51	2·94	2·83

Significant differences ·24 (P = ·01)
·18 (P = ·05)

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

126	113	112	130	122	124	119	121

Significant differences 34 (P = ·01)
26 (P = ·05)

In the three experiments listed in Table 3 there was a marked increase in growth with Mo but no effect on the concentration of ascorbic acid. In (3) there were also responses to Zn ($P < ·05$) and (KMnB) ($P < ·01$) but again the ascorbic acid concentration was unaffected. The lucerne was grown for 8 weeks and the subterranean clover for 12 weeks.

TABLE 4.

Yield and ascorbic acid concentration of subterranean clover grown on a grey brown sand from Lake Albert, S.A.

(1) Subterranean clover (sown 14th April). Means of 2 cultures.

(a) Yield (gm.).

	—	Cu	Zn	K	Mo	CuZn	CuK	CuMo
L_1	2·01	2·01	2·18	1·94	2·28	2·98	2·07	1·99
L_2	1·73	1·57	2·04	1·77	1·66	2·25	1·82	1·70

	ZnK	ZnMo	KMo	CuZnK	CuZnMo	CuKMo	ZnKMo	CuZnKMo
L_1	2·99	2·73	2·71	2·51	2·69	2·16	3·16	2·73
L_2	2·64	2·47	1·94	2·49	2·58	1·78	2·71	2·35

Significant differences 1·03 (P = ·01)
·77 (P = ·05)

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

	—	Cu	Zn	K	Mo	CuZn	CuK	CuMo
L_1	109	109	112	109	109	101*	95*	109*
L_2	101	99	101	101	102	96*	80*	88*

* One replicate.
L_1 = normal day length (ranging from 10¾ to 12¾ hours). L_2 = shortened day (8 hours).

(2) Subterranean clover (sown 16th March). Means of 2 cultures.

—	(CuZn)	K	(MnB)	Mo
(a) Yield (gm.).				
1·49	1·92	2·39	1·59	1·64

Significant differences ·73 (P = ·01)
·55 (P = ·05)

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

125	120	110	153	128

Significant differences 60 (P = ·01)
45 (P = ·05)

(3) Subterranean clover (sown 29th May). Means of 5 cultures.

—	(KMnB)	Zn
(a) Yield (gm.).		
·93	1·10	1·52

Significant differences ·23 (P = ·01)
·17 (P = ·05)

(b) Ascorbic acid concentration (mg. per 100 gm. dry matter).

82	82	107

Significant differences 14 (P = ·01)
10 (P = ·05)

Experiment (1), Table 4, was sown in the spring and harvested eight weeks later. This included a comparison between an artificial day length of 8 hours and normal light duration of 10¾ to 12¾ hours. With the longer day there was an increase both in the yield and the concentration of ascorbic acid ($P < \cdot01$); but although the yield was increased by Zn ($P < \cdot01$), K and Mo ($P < \cdot05$), none of these elements affected the ascorbic acid concentration.

Experiment (2) was sown in autumn and grown for twelve weeks; neither yield nor ascorbic acid concentration was affected by the treatments.

In experiment (3), which was winter sown and grown for ten weeks, both yield and the concentration of ascorbic acid were increased by the application of Zn ($P < \cdot01$).

TABLE 5.

Summary of responses in yield and ascorbic acid concentration of subterranean clover to Zn, excluding those experiments in which Zn formed part of a group of elements employed as one unit treatment.

Time of sowing.	Age at harvest.	Table in text.	Yield (gm.). —	Yield (gm.). Zn.	Significance.	Ascorbic acid concentration (mg. per 100 gm. dry matter). —	Ascorbic acid concentration (mg. per 100 gm. dry matter). Zn.	Significance.
16th May	12 weeks	3 (3)	2·37	2·25	—	122	120	—
29th „	10 „	4 (3)	·93	1·52	**	82	107	**
31st „	12 „	6	·73	1·01	**	110	130	**
21st July	7 „	2 (2)	·56	·68	**	76	102	**
„ „	8 „	2 (2)	1·06	1·47	**	91	106	**
„ „	9 „	2 (2)	1·15	1·68	**	82	93	**
„ „	10 „	2 (2)	1·61	2·33	**	92	85	—
„ „	11 „	2 (2)	2·64	3·11	**	125	118	—
14th Aug.	8 „	4 (1)	1·94	2·59	**	105	107	—

** Significant $P < \cdot01$.

TABLE 6.

Yield and ascorbic acid concentration of four different legumes grown on grey brown soil from Lake Albert (same as in Table 4).

	Yield (gm.). (sown 31st May). Means of 2 cultures. Peas.	Barrel medic.	Sub-clover.	Strawberry clover.	Ascorbic acid concentration (mg. per 100 gm. dry matter). Peas.*	Barrel medic.	Sub-clover.	Strawberry clover.
—	3·03	·64	1·45	·55	300	193†	118	290
Cu	2·88	·55	1·37	·46	255	180†	113	300
Zn_6	3·15	1·45	1·76	·58	293	170†	133	348
Zn_{12}	3·05	1·66	1·74	·55	295	194†	133	365
Zn_{18}	2·96	1·35	1·56	·46	280	189†	125	348
Mo	3·18	·65	1·71	·45	253	186†	118	260
$CuZn_6$	3·25	1·59	1·82	·52	273	187†	133	330
$CuZn_{12}$	3·04	1·60	1·84	·54	295	189†	128	328
$CuZn_{18}$	2·84	1·58	1·86	·50	275	183†	138	338
CuMo	3·22	·58	1·34	·50	258	169†	100	275
Zn_6Mo	3·37	1·63	2·10	·83	265	180†	143	330
$Zn_{12}Mo$	3·69	2·14	2·19	·71	293	175†	138	353
$Zn_{18}Mo$	3·69	2·00	2·04	·86	275	187†	133	330
$CuZn_6Mo$	3·17	2·14	2·27	·90	265	178†	135	343
$CuZn_{12}Mo$	3·56	2·47	2·28	·82	260	161†	130	325
$CuZn_{18}Mo$	3·40	2·36	2·20	·89	270	162†	125	343

* 3 missing values estimated for peas. † 1 replicate.

Significant differences for	Yield. $P = \cdot01$.	Yield. $P = \cdot05$.	Ascorbic acid content. $P = \cdot01$.	Ascorbic acid content. $P = \cdot05$.
Peas	·98	·73	64	48
Barrel medic	1·01	·76	27	20
Sub-clover	·54	·41	30	22
Strawberry clover	·21	·16	91	69

There was a response in growth by all four to Mo ($P < \cdot 01$) and by all but peas to Zn ($P < \cdot 01$). The ascorbic acid concentration was unaffected by Mo with any of the four plants but an increase with Zn was obtained in the cases of subterranean clover and strawberry clover.

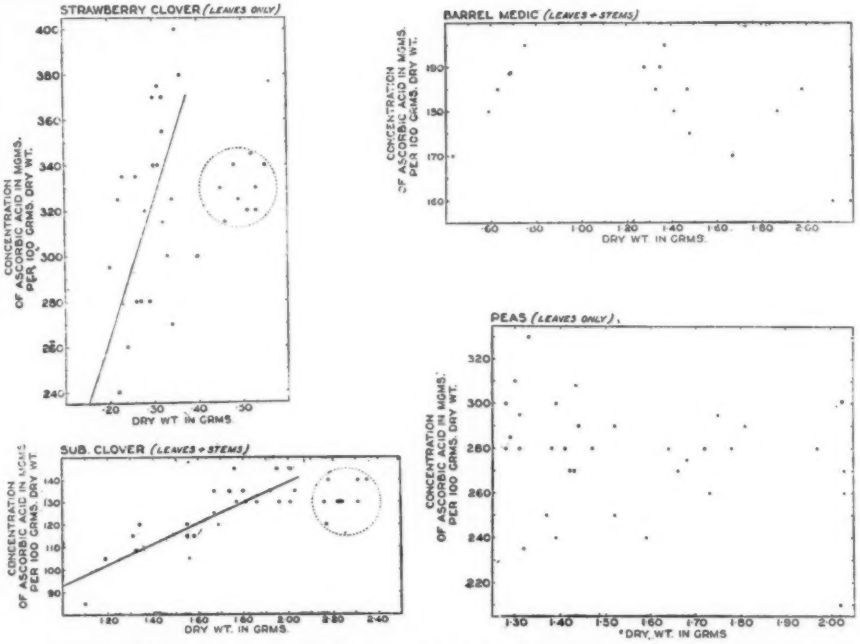
STRAWBERRY CLOVER (LEAVES ONLY)
BARREL MEDIC (LEAVES + STEMS)
SUB. CLOVER (LEAVES + STEMS)
PEAS (LEAVES ONLY)
CONCENTRATION OF ASCORBIC ACID IN MGMS. PER 100 GRMS. DRY WT.
DRY WT. IN GRMS.

Fig. 2. Relationship between yield and ascorbic acid concentration for the four legumes of Table 6. The regression lines given for subterranean clover and strawberry clover represent the relationship for yield $< 2 \cdot 05$ gm. and $< \cdot 375$ gm. respectively. The points surrounded by the dotted circles have not been included in the calculation of the regression lines.

DISCUSSION.

Silver beet and lettuce, sown early in the season when conditions of both light and temperature were favourable, were unaffected either in terms of yield or ascorbic acid concentration. This applies to zinc, potassium and molybdenum in the case of lettuce, and zinc, potassium and copper in the case of beet. The soils used had responded to these elements when applied to herbage legumes as test plants (Table 1).

Although silver beet failed to respond in these tests, subterranean clover grown on similar soil later in the season gave significant responses both in the yield and the ascorbic acid concentration, to applications of zinc (Table 2). Both the lower sensitivity of the leaf vegetable to micronutrient deficiency and the more favourable conditions of light and temperature during their period of growth are probably concerned in this difference.

In several experiments, molybdenum produced a marked increase in growth (Table 3 (1), (2), (3); Table 4 (1) and Table 6), but in no case was there any effect on the concentration of ascorbic acid. There were three responses of the yield to potash, Table 2 (2), at the first and second harvests, and Table 4 (1), but the ascorbic acid concentration was again not affected.

In all the experiments conducted, the only cases in which significant increases in the concentration of ascorbic acid were obtained were associated with the application of zinc on the one hand, and development of the test plants under winter conditions of comparatively low light intensity and a lower temperature than in autumn and spring, on the other.

Work in progress at the Waite Institute over several seasons has shown that the response of herbage legumes to zinc may be negligible in autumn and spring, whereas marked differences may be obtained on the same soil during the winter months, this being due to a failure in the growth of the control plants. The climatic factors concerned are being investigated. Among these factors, length of day has received particular attention. Table 4 (1), shows that both the yield and the ascorbic acid concentration of subterranean clover increase as the duration of daylight is lengthened; but changes in the length of day have had no effect on the response of the content of ascorbic acid to the application of zinc. It thus appears that although there is a strong interaction between yield response to zinc and length of day, there is no such interaction in the case of the ascorbic acid content.

Table 5 lists the zinc effects in chronological order. In the first experiment there was no increase in either the yield or the concentration of ascorbic acid with added zinc. The last two harvests of 2 (2) and 4 (1) show responses in the yield, but not in the ascorbic acid concentration. This effect could be interpreted as being due either to the time of the year or the stage of maturity of the plant. In the earliest experiment, in which negative results were obtained, the plants were more advanced at harvest.

In the experiment with subterranean clover appearing in Table 2 (2), subterranean clover sown on the 21st July was harvested at successive growth stages seven, eight, nine, ten and eleven weeks from seeding, with a view to following the development of the ascorbic acid content as the plant advanced in its maturity.

Examination of Fig. 1 shows a close positive linear relationship between the yield of a plant and its ascorbic acid concentration at the three harvests ($P < \cdot.001$), the slope becomes less steep at each harvest and at the fourth harvest is negative. For the fourth harvest the regression line is significant only at the 5 p.c. level and at the fifth harvest there is no significant relationship.

In the above experiment there is still a variation in both length of day and light intensity with the successive changes of maturity, but in a further experiment (Table 6), four legumes with differing rates of growth were grown under identical conditions of light and of all other environmental factors. The order in which maturity was most rapidly obtained in the four legumes employed was as follows: peas, barrel medic, subterranean clover (Mt. Barker type) and strawberry clover (Palestine type), the strawberry clover and subterranean clover being considerably slower in their rate of development than the barrel medic and the peas.

The yield (Table 6) was increased by zinc in the case of all but the peas. The ascorbic acid concentration was improved in the case of strawberry clover and the subterranean clover, but not in the case of barrel medic and the peas. Fig. 2 shows a positive linear relationship between the yield and the concentration of ascorbic acid in the case of the two clovers, so long as the yield is below a certain critical point, but above this point it breaks down. It would appear that in the case of peas and barrel medic, this critical point had already been passed prior to harvest, with a resulting slight downward trend.

SUMMARY.

Silver beet, lettuce, peas and several herbage legumes were grown in pot cultures on soils which had previously shown evidence of marked deficiency in potash, zinc and molybdenum.

There was no increase in the yield, or any modification of the ascorbic acid content of the two leaf vegetables, following applications of the deficient nutrients.

The herbage legumes responded significantly to all three minerals in terms of their yield; but the concentration of ascorbic acid was affected only by the application of zinc and then only at certain harvests.

The yield responses to molybdenum were particularly marked; those to potash were smaller by comparison. In neither case was there any evidence of any increase in the ascorbic acid content.

Increases in yield due to zinc were marked during the winter months, but were negligible in both autumn and spring. Responses in yield to zinc were not necessarily accompanied by increases in ascorbic acid. Where there was no response of the yield to zinc there was also no increase in the ascorbic acid content.

Increases in ascorbic acid concentration were in all cases associated with an early growth stage and were most evident in those plants that matured relatively slowly.

It is concluded that modification of the ascorbic acid content by treatment with zinc is associated with the stage of maturity of the plant.

Acknowledgments. We wish to thank Professor H. C. Trumble for advice and helpful criticism and also Dr. J. W. H. Lugg for assistance in the method of vitamin estimation.

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THE EFFECT OF STORAGE ON THE RIBOFLAVIN CONTENT OF URINE

by E. C. SLATER AND D. B. MORELL

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(Accepted for publication 23rd March, 1946.)

It is often necessary to keep urines for several weeks, or even months, before measuring their riboflavin content. It is, therefore, desirable to know the most suitable conditions of storage so that no riboflavin is lost.

The present paper reports the results of a study of the loss of riboflavin when urine is stored under various conditions of temperature and pH.

EXPERIMENTAL.

The following samples of urine were analysed: A, from rats fed a riboflavin-free diet for 5 weeks after weaning; B, from rats fed a normal colony diet; C1, C2, C3, from a normal human on an ordinary diet.

The rat urines were considerably diluted by the water used to wash the sides of the metabolism cage. Urines A, B and C3 were adjusted to pH 1·0, urine C2 was adjusted to pH 4·0 and urine C1 was kept kept at its natural pH (6·5).

Each of the urines was analysed on the day of collection and again after storage under toluene in amber bottles at room temperature for various periods. Portions of urine C3 were kept at 4° C. and 37° C. as well as at room temperature.

Riboflavin was determined by a specific method described by Slater and Morell (1946).

RESULTS.

Human urines. The results, given in Fig. 1, show that urine C1 stored at room temperature at pH 6·5, suffered no appreciable loss of riboflavin after 246 days. On the other hand, urine C2, which was stored at room temperature at pH 4·0, showed an immediate loss of riboflavin, amounting to 17 p.c. after 87 days; thereafter, there was no appreciable change during storage for an additional 126 days. Urine C3, stored at pH 1·0 at three different temperatures, gave some interesting results. The urine kept at room temperature throughout the storage period (228 days) behaved similarly to urine C2. It lost 15 p.c. of its riboflavin in 51 days and then showed little change. The sample kept at 4° C. showed a much more rapid loss of riboflavin (15 p.c. in 7 days, 29 p.c. in 93 days). When subsequently stored at room temperature, the riboflavin content increased and, when analysed after 229 days' total storage, it contained the same amount of riboflavin as the sample kept at room temperature over the entire period of storage. The sample kept at 37° C. showed only a slight loss (9 p.c. after 93 days). When subsequently removed to room temperature, there was a more rapid fall of the riboflavin content and the value after 228 days' total storage was identical with that of the other two samples. The results for urine C3 clearly showed that the riboflavin content depends upon the temperature of storage. The same final figures were obtained for samples eventually kept under the same conditions, even though intermediate temperatures ranged from 4° C. to 37° C.

It was found that the loss of riboflavin during the storage of these urines was due to adsorption of riboflavin on the uric acid which crystallized from the urine. This finding offered a complete explanation of the results discussed above since conditions favourable to the crystallization of uric acid, such as a low pH and a low temperature, caused the greatest loss of riboflavin. The rise in the riboflavin content when urine C3 (stored at 4° C.) was transferred to room temperature was probably mainly due to the following factors:

1, solution of some of the uric acid with consequent release of the adsorbed riboflavin;
2, a greater degree of adsorption of riboflavin on the uric acid at the lower temperature.

Urine C1, which showed practically no loss, contained a small amount of a fine precipitate, quite unlike uric acid.

Direct evidence that the crystals of uric acid did contain riboflavin in amounts sufficient to explain the losses was obtained as follows. The crystals, which had separated from the sample of urine C3 stored at 4° C. and then at room temperature, were collected quantitatively, dried and weighed. The crystals gave a positive murexide reaction and, under the microscope appeared as a homogeneous collection of deeply pigmented crystals, similar to the "barrel-shape" crystals described by Hawk and Bergeim (1938) as a form of uric acid. The crystals were soluble in conc. H_2SO_4, but not in glacial acetic acid. A sample was dissolved in conc. H_2SO_4, neutralized, and diluted with water and the riboflavin content of the solution determined. It was found that

the crystals contained 170 μg./gm. riboflavin and this accounted for 69 p.c. of the riboflavin lost from the urine (which amounted to 22 p.c. of the original riboflavin content).

These findings show that if a sample of human urine must be stored for some time before the riboflavin is determined, conditions favourable to the separation of uric acid (low urinary volumes, low temperatures and low pHs) should be avoided, if possible.

Our results do not agree with those of Hagedorn *et al.* (1945), who found no significant loss in the riboflavin content of 180 samples of urine after storage in a refrigerator for up to three weeks. These urines had been acidified by adding 10 ml. glacial acetic acid to each 24 hour sample.

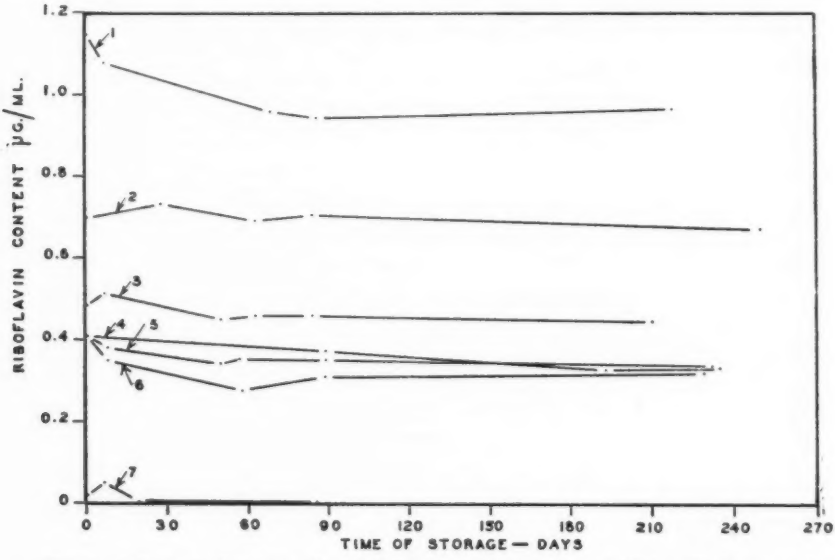

Fig. 1. Effect of storage on the riboflavin content of urine. 1. Urine C2, human urine, stored at pH 4·0 at room temperature; 2. Urine C1 (× 2), human urine, stored at pH 6·5 at room temperature; 3. Urine B, rat urine, stored at pH 1·0 at room temperature; 4. Urine C3, human urine, stored at pH 1·0 at 37° C. for 90 days, then at room temperature; 5. Urine C3, stored at pH 1·0 at room temperature; 6. Urine C3, stored at pH 1·0 at 4° C. for 90 days, then at room temperature; 7. Urine A, urine from riboflavin-deficient rat, stored at pH 1·0 at room temperature.

Rat urines. Urine B, a rat urine kept at pH 1·0 at room temperature showed a rather smaller loss than the similar human urine, viz. 8 p.c. after 84 days, 11 p.c. after 210 days. Urine A2, which initially contained a barely measurable amount of riboflavin, contained none after 20 days' storage.

The explanation of these losses cannot be the same as the one given for human urines, because rats do not excrete an appreciable amount of uric acid. Possibly, the small loss was due to adsorption on an unidentified fine precipitate in these urines.

SUMMARY.

The riboflavin content of human urine kept under acid conditions decreases during storage, due to adsorption on the uric acid which crystallizes from the urine. A sample of this uric acid contained 170 μg./gm. riboflavin.

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THE INFLUENCE OF FOOD ON THE SIZE OF TWO STRAINS OF *CALANDRA ORYZAE* L.

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(Accepted for publication 13th March, 1946.)

Some samples of *Calandra oryzae* breeding in maize and collected from Atherton, Queensland, and Northern New South Wales were found to have a mean length (elytrae and pronotum) of 3·2 mm. and 3·3 mm. respectively. These figures are significantly greater than the figure 2·9 mm. which was the mean length of the "large strain" which had been breeding for several generations in wheat before the measurement was made (Birch 1944). Another sample of *C. oryzae* breeding in maize and collected in Sydney measured 2·7 mm. in length. This is significantly larger than the figure 2·5 mm. which is the length of the "small strain" when bred in wheat for several generations (Birch 1944) but it is smaller than the "large strain" when bred in wheat.

Experiments were done to see whether these insects represented further strains of *C. oryzae* or whether their different size was due to their having been bred in maize instead of wheat.

The two strains described previously (Birch 1944) are referred to in this paper as "large" and "small". The specimens breeding in maize from the field are designated "Atherton" and "Sydney".

Crossing Experiments.

The insects from Atherton and Sydney were bred in wheat for one generation and the following crosses made:

Atherton ♂ × "small" strain ♀	(infertile)
Small strain ♂ × Atherton ♀	(infertile)
Atherton ♂ × "large" strain ♀	(fertile)
Atherton ♂ × "large" strain ♀	(fertile)
Sydney ♂ × Atherton ♀	(infertile)
Atherton ♂ × Sydney ♀	(infertile)

The Table shows that the Atherton insects were interfertile with the large strain but not with the small strain suggesting that the Atherton insects are large strain which have evidently become larger by being bred in maize. The fact that the "Atherton-Sydney" cross was infertile suggests that the Sydney specimens were small strain which had become larger by being bred in maize.

The Size of the Large and Small Strains Bred in Wheat and Maize.

The two strains were bred for seven generations at 30° C. in both wheat and maize of 14 p.c. m.c. Measurements were then made of the total length of pronotum and elytrae and of the maximum width of 50 insects. The same procedure was carried out with the Atherton specimens. The Sydney specimens were measured after having passed through one generation in wheat.

[1] This work was carried out with the aid of a research grant from the Federal grant to the University of Adelaide.

TABLE 1.

Showing the effect of wheat and maize on the size of Calandra oryzae.

Strain.		Size in wheat in mm. Total length.	Max. width.	Size in maize in mm. Total length.	Max. width.
"Small" strain	7 gens	2·46	0·95	2·72	1·07
"Large" strain	7 gens	2·87	1·12	3·23	1·34
	1 gen	—	—	3·30	1·40
"Atherton" (large strain)	7 gens	2·84	1·20	3·32	1·31
	1 gen	3·27	2·33	—	—
Sydney	1 gen	2·54	1·10		

At P. 0·01 significant differences of log $(\times + 1) = 0{\cdot}01$ where $\times$ = mean values shown in Table.

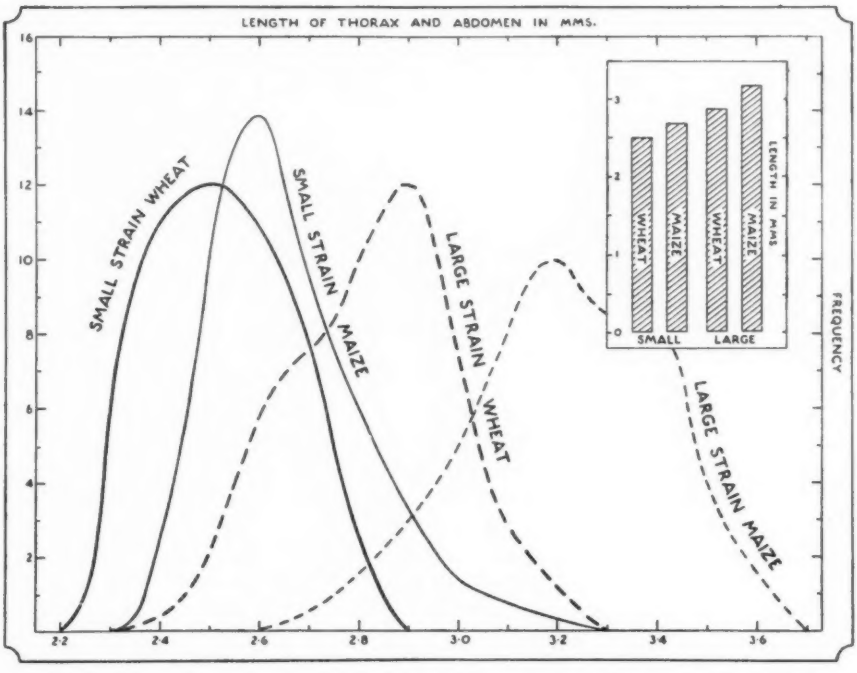

Fig. 1. Frequency curves showing the size distribution of populations of 50 individuals of the "large" and "small" strains when bred in wheat and maize. Inset diagram shows the mean lengths.

The insects from Atherton bred in wheat were reduced to the same size as the "large" strain when bred in wheat; and the "large" strain bred in maize became as large as the Atherton insects when bred in maize, thus confirming the identity in size of these two lots of insects. The small strain also increased in size when bred in maize but remained 0·2 mm. shorter than the large strain bred in wheat (significant difference = 0·1 mm.). The percentage change in size is the same for the two strains, i.e. there is no interaction between strain and food type. These differences are illustrated in Fig. 1 (inset). The extent to which the populations of the large and small strains became similar in size when bred in wheat and maize respectively is shown in the frequency diagrams in Fig. 1.

In the cases in which the size was measured in the first generation the change in size in one generation is seen to be about the same as the total effect of seven generations, except for the Atherton specimens when bred for one generation in wheat after maize. The reason for this latter result is not known.

DISCUSSION.

In 1855 Motschulsky recorded the existence of a large form of *C. oryzae* in maize and regarded it as a new species *C. zea-mais* Motsch. on the basis of size and sculpturing on pronotum and elytrae (Zumpt F. 1935). Zumpt considers that the size and sculpturing are too variable to enable an exact separation of the two forms to be made. Furthermore, he found that the male genitalia were indistinguishable, whereas the other species of *Calandra* were separable on this basis. He proposed, however, that three races should be distinguished, a race in rice, one in wheat and one in maize, and he states that the differences between the races are both of size and sculpturing of the pronotum and elytrae. A smaller form from rice has been described from Japan as *C. sasakii* Takahashi (Takahashi 1928). Kinoshita and Ishikura (1940) state that *C. oryzae* from rice becomes larger when bred on dried sweet potato, and Richards (1944) states that the size of *C. oryzae* is increased by breeding in maize. Due to the kindness of the Chief of the Bureau of Entomology and Plant Quarantine of the United States Department of Agriculture, the author has been able to examine a series of *C. oryzae* from the Bureau's collection. These show a distinct gradation in size according as the specimens came from rice, wheat or maize.

It is not now surprising that there has been considerable confusion in the synonomy of *C. oryzae* seeing that there are at least two strains involved each of which has a different size in different cereals.

SUMMARY.

Two strains of *Calandra oryzae* which appear morphologically identical can be distinguished by their size. After breeding in wheat for 7 generations the large strain measured 2·87 mm. from the pronotum to the tip of the elytra: the small strain measured 2·46 mm. After breeding for 7 generations in maize the large strain measured 3·23 mm. and the small strain 2·72 mm. The change in size due to change of food was relatively the same in both strains, i.e., there was no interaction between strain and food.

Acknowledgment. The author acknowledges the help of Mr. H. G. Andrewartha who encouraged him to pursue the investigation.

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MUMPS

2. VIRUS HAEMAGGLUTINATION AND SEROLOGICAL REACTIONS

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(Accepted for publication 17th January, 1496.)

Levens and Enders (1945) reported that amniotic fluid of chick embryos infected with mumps virus caused agglutination of fowl red cells. This observation has greatly facilitated laboratory work with mumps. Investigations described in this paper show that the agglutination is probably caused by the mumps virus itself and so is similar to that caused by the influenza viruses and the virus of Newcastle disease.

METHODS.

The technique we use for titration of virus is the one used in this laboratory for titration of influenza virus (Burnet, Beveridge, Bull and Clark, 1942). Red cells are obtained from fowls selected as having cells which give high haemagglutination titres with mumps virus. The virus suspension is diluted in twofold serial dilutions in volumes of 0·25 ml. in tubes 3" × ½". An equal volume of 2·0 p.c. fowl red cells is added and then a further 0·25 ml. of saline. After a thorough shaking the cells are allowed to settle at room temperature and the readings made from the pattern of cells on the bottom of the tube. An unbroken hemisphere of completely agglutinated cells is designated "++" and the end point is taken as the dilution giving a central button of agglutinated cells surrounded by a thin, even zone of agglutinated cells ("+"). More clear-cut results are obtained at room temperature than at 37° C. or at 4° C.

For titration of inhibition of agglutination serial dilutions of serum are made and five times the concentration of virus required to give a "+" reaction is added to each tube. Formalinized virus is used to avoid working with active virus unnecessarily. Red cells are added and end point is taken as the tube giving partial agglutination. When fowl cells are used the end point usually cannot be read with any accuracy because in those tubes in which there is no inhibition the agglutinated cells slip from the rounded bottom of the tube into a clump in the centre so that it is difficult to see whether or not there is agglutination. This slipping is caused by the presence of dilute serum, for control tubes give the usual type of agglutination in which the cells adhere to the hemisphere of glass forming the bottom of the tube. The difficulty is avoided by using guinea-pig cells but first it is necessary to treat the serum (diluted 1: 10) with 10 p.c. packed guinea-pig cells to absorb out the agglutinins against guinea-pig cells which most human sera contain.

RANGE OF CELLS AGGLUTINABLE.

To compare the agglutinability of red cells from different fowls, suspensions were prepared from the cells of 33 fowls varying in age from 6 months to 2 years. When the same virus preparation was titrated simultaneously with each of these suspensions, the titres varied from 25 to 100. Corresponding results were obtained with each of the three strains of virus. With influenza A and B viruses there was some variability in titre obtained with suspensions of different cells but these variations did not correspond with those shown by the three strains of mumps virus nor were they as great.

The differences in titre obtained with different cells were more marked with weak virus preparations than with strong ones. With the former, differences as great as from 60 to less than 10 were shown with cells that only gave differences of 160 to 80 with stronger preparations. Cells obtained from the same fowls on several occasions showed no alteration.

Twenty suspensions of cells prepared from 8 13-day embryos and from 12 2-day-old chicks gave results essentially similar to those obtained with cells from adult fowls.

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[1] This work was carried out under a grant from the National Health and Medical Research Council.

Human red cells were obtained from 16 persons, 4 from each of the main blood groups. Suspensions of these gave titres of 30 to 50 with a virus suspension which had a titre of 300 with one of the more sensitive fowl cell suspensions.

Several guinea-pig cell suspensions have been tested and found to give titres in the same range as fowl cells.

Pigeon cells were agglutinated but to a lower titre than fowl cells. Virus suspension with a titre of 600 with sensitive fowl cells had a titre of 140 with pigeon cells and a weaker preparation produced no agglutination.

PROPERTIES OF THE HAEMAGGLUTININ.

When amniotic fluid virus was heated at 56° C. for 20 minutes the haemagglutinin was destroyed. Holding at 37° C. for up to 7 hours after addition of 0·1 p.c. formalin caused only a trivial reduction in agglutinating titre but after 24 hours at 37° C. there was a marked reduction. Amniotic fluid virus with up to 0·2 p.c. formalin showed no fall in titre when kept 4 weeks in the refrigerator. Untreated amniotic fluid virus held in the refrigerator maintained its titre for at least several days but at 37° C. some fall was noticeable after 4 hours. When amniotic fluid virus was shaken with ether the titre was reduced from 200 to 50.

As with influenza virus, the haemagglutinin is removed from the virus suspension by absorption with red cells. After 3½ hours at 37° C. most of the virus is eluted from the red cells and can be recovered. The higher the titre of the virus the more rapid the elution. Cells from which the virus has been eluted are no longer agglutinable by mumps virus. Burnet (1946) has recently shown that cells rendered insusceptible to agglutination by influenza or Newcastle disease viruses by adsorption and subsequent elution of an influenza virus are also insusceptible to agglutination by mumps virus. The mumps virus causes a considerable amount of haemolysis of the cells during the eluting process.

A quantitative absorption and elution experiment was carried out. Amniotic fluid virus was diluted 1: 2 in saline and sufficient fowl cells to make a 10 p.c. suspension were added. This was held at 20° C. for 30 minutes. The cells were spun down and resuspended in saline to the original volume and placed in a water bath at 37° C. The suspension was shaken every hour and removed after 3½ hours and the cells spun down. The various fractions were titrated by haemagglutination and by complement fixation and the results are shown in Table 1. The dilutions are expressed in terms of the original amniotic fluid. Most of the haemagglutinin was absorbed on the cells and recovered in the eluate, as also was most of the complement-fixation antigen. When this experiment was repeated similar figures were obtained for the haemagglutinin, but less complement fixing antigen was removed by absorption and less recovered in the eluate.

TABLE 1.

Absorption and elution of virus from red cells.

Fraction.	Haemagglutination titre.	Complement-fixation.				
		Dilution of antigen.	Serum dilution. 1/5	1/15	1/45	1/135
Original fluid	320	1/15	4	4	3	3
		1/20	4	3	2	1
Supernatant after absorption	30	1/10	2	–	–	–
Eluate	240	1/5	4	4	3	3
		1/10	4	3	1	1

Under complement-fixation the figures represent the degree of fixation, for example 4= complete fixation.

According to filtration experiments done by Habel (1945) the mumps virus is of a size which should be deposited by centrifugation at 15,000 r.p.m. Two similar experiments were done to determine whether the agglutinin could be deposited at this speed. Virus in amniotic fluid was diluted 1: 3 in saline and centrifuged at 2,500 r.p.m. for 5 minutes to throw down gross particles and then spun at 15,000 r.p.m. for 1 hour. Samples of the original fluid, the supernatant and the resuspended deposit were titrated by haemagglutination and by complement fixation. The results are shown in Table 2. The dilutions are expressed in terms of the original amniotic fluid. In both experiments the bulk of the agglutinin and of the complement-fixing antigen were removed by centrifugation at 15,000 r.p.m.

TABLE 2.

Centrifugation of mumps virus.

Fraction.	Haemagglutination titre.	Complement-fixation.				
		Experiment I.				
		Dilution of antigen.	Serum dilutions. 1/5	1/15	1/45	1/135
Original fluid	300	1/15	4	4	4	4
Supernatant	40	1/5	1	–	–	–
Re-suspended deposit	200	1/10	4	4	3	1
		1/15	–	–	–	–
		Experiment II.				
Original fluid	320	1/15	4	4	3	3
		1/20	4	3	2	1
Supernatant	60	1/3	3	–	–	–
		1/5	2	–	–	–
Re-suspended deposit	200	1/5	4	4	4	3
		1/10	2	–	–	–

These experiments with centrifugation and red cell absorption and elution gave results essentially similar to those obtainable with influenza virus and indicate that in all probability the agglutinin is the virus itself. The results also indicate that most of the complement-fixing antigen is the virus itself although possibly there is some antigen which fixes complement but does not cause agglutination. Since heating the virus suspended at 56° C. or 20 minutes destroys the agglutinin without affecting the complement-fixation titre, any virus preparation might be expected to contain some degenerated virus capable of fixing complement but not of causing agglutination.

NONSPECIFIC INHIBITION.

Normal allantoic fluid and yolk sac suspension have an inhibitory effect on haemagglutination by the mumps virus. The addition of normal allantoic fluid from 10- to 11-day embryos or 10 p.c. yolk sac suspension to give a final concentration of 33 p.c. in each tube reduced the titre of a virus suspension from 900 to 200 and with weaker virus fluids agglutination was entirely inhibited. When allantoic fluids from uninfected embryos were tested for inhibitory effect using essentially the same technique as for serum neutralization experiments with 5 agglutinating doses of virus, fluid from embryos 10 to 15 days old gave inhibition titres mostly in the range of 4 to 8, but occasionally as high as 40. There was variation from embryo to embryo of the same age but the highest titres were obtained with fluids from the oldest ones.

Inhibition is also shown by suspension of chorioallantoic membrane and by amniotic fluid from embryos 13 to 16 days old but not by amniotic fluid of 10-day embryos or by yolk fluid. The inhibitory effect is shown with guinea-pig cells as well as with fowl cells. Heating of allantoic fluid at 56° C. for 30 minutes or incubation of a mixture of virus and allantoic fluid at 37° C. for 4 hours before titration does not lower the inhibition titre.

The same inhibition titre was obtained with allantoic fluid when the cells were added immediately after the virus and allantoic fluid were mixed as when the mixtures had stood an hour at room temperature or overnight in the refrigerator before adding the cells.

The inhibitory effect is lost rapidly on dilution and the law of multiple proportions does not apply to this phenomenon. Thus it is possible to have a mixture which is neutral, that is, shows no agglutination at low dilution but when diluted out does give agglutination. Amniotic fluid virus suspension with a titre of 80 was mixed with equal parts allantoic fluid with a high inhibitory titre and the mixture diluted in series. There was only slight agglutination at 1: 5 and 1: 10 whereas at 1: 20 to 1: 80 agglutination was much stronger. These observations show that with infected allantoic fluids or yolk sac suspensions agglutination would be completely inhibited in fluids which otherwise would give a low titre, but in potent virus suspensions the inhibitory substance would not affect the titre.

Nonspecific inhibition is sometimes shown with serum containing no specific antibody, for instance, normal rabbit serum or human serum from a person with no history of mumps. Different strains of virus vary considerably in this regard. With strain WAL the majority of negative human sera have a titre of less than 10 and the remainder from 10 to 30, whereas with STO and IND they have titres of 20 to 80. Previous heating of human serum at 60° C. for 20 minutes or rabbit serum at 62° C. for 20 minutes considerably reduces the nonspecific inhibition titre but causes little or no reduction in the titre of specific antibody.

SEROLOGICAL REACTIONS.

Sera from several mumps convalescent patients have haemagglutinin inhibition titres between 80 and 320. Sera from persons who had had mumps many years previously gave the same titres as those giving no history of mumps, i.e. from < 10 to 30. One case of mumps encephalitis from which acute and convalescent specimens were obtained showed a change in titre from 20 to 80.

The three strains of virus were compared by titrating them against two homologous convalescent human sera and several other convalescent human sera. There was no serological difference between the 3 strains.

Each strain of virus was inoculated intravenously into two rabbits. A single dose of 1 ml. of active virus was given. Blood was taken three weeks after inoculation and the sera titrated by haemagglutination with guinea-pig cells after heating at 62° C. and absorbing with guinea-pig cells. The results are shown in Table 3. The titres of the sera taken before inoculation are due to nonspecific inhibition. All sera taken after inoculation showed a considerably higher titre and this is regarded as due to specific antibody. No differences were revealed beyond the normal slight variability from animal to animal and it is concluded that these three strains of virus are identical serologically.

TABLE 3.

Cross serology with rabbit sera.

	Serum.											
	WAL.				STO.				IND.			
Virus.	SI	SII	SI	SII	SI	SII	SI	SII	SI	SII	SI	SII
WAL	30	160	20	140	30	290	30	80	30	80	40	150
STO	30	110	20	100	70	320	40	80	20	100	80	160
IND	30	260	20	160	70	>320	60	120	30	80	80	320

The figures are the inhibition of haemagglutination titres. SI = serum taken before inoculation, SII = serum taken 3 weeks after inoculation. Two rabbits were inoculated with each strain.

Some preliminary experiments on vaccination of human subjects have been done. Yolk sac suspensions have not been used owing to the large amount of non-virus material contained and as yet it has not been possible to produce allantoic fluid virus of sufficiently high titre. The best virus suspension has been amniotic fluid harvested after 6 or 7 days' incubation at 34° C. following inoculation into the yolk sac of 7 or 8-day embryos. Strain WAL has been much better than the other two for producing fluids of good titre. With this strain each egg usually yields 1 to 2 ml. of fluid of very low protein content and of high titre.

TABLE 4.

Titrations of sera from vaccinated persons.

	SI	SII	SIV
F.B.	<10	10	15
P.L.	10	10	30
McA.	10	15	40
McC.	10	120	100
S.A.	10	160	—
M.K.	15	70	80
L.H.	15	60	40
W.B.	40	80	70
J.S.	—	—	300
B.R.	—	—	320

SI = serum taken at time of first inoculation. SII = serum taken 2 weeks after first inoculation. SIV = serum taken 2 weeks after second inoculation. J.S. and B.R. = convalescent sera after clinical mumps.

The virus was inactivated by 0·1 p.c. formalin at 37° C. for 4 hours. One batch of vaccine was found to contain active virus when it had been held at 37° C. for only 2 hours after addition of the formalin as did another batch which was held 6 days at 4° C. and not at 37° C. at all. The vaccine retained its agglutinin titre unaltered during 4 weeks at 4° C.[2]

[2] Since this paper was sent to the press we have used vaccine made from allantoic fluid with a haemagglutination titre in the vicinity of 100. The virus is formalinized at 0·1 p.c. and held for 12 hours at 37° C.; occasional batches were found to contain active virus after 4 hours at 37° C.

This vaccine was injected subcutaneously in 1 ml. amounts into 8 young adults, only two of whom (L.H. and W.B.) had had mumps. Specimens of serum were taken at the time of inoculation and two and three weeks later. Seven of the subjects received a second inoculation 4 weeks after the first and were bled again two weeks later. These sera were titrated by haem-agglutination-inhibition using guinea-pig cells after preliminary adsorption to remove agglutinins against these cells. The results of these tests are shown in Table 4. The titres obtained with the serum taken three weeks after the first inoculation were the same as those of the sera taken at two weeks so they have been omitted from the Table. Included at the bottom of the Table are the values for two sera from persons recently recovered from mumps for comparison.

Five of the eight subjects had appreciable increases in titre as a result of one inoculation and the four of these who were given a second inoculation had no further increase. The other three, those listed first in the Table, had no appreciable increase after the first inoculation but had some increase after the next injection. Possibly these three were the only ones who had no basic immunity to mumps and all those in the other groups may have either had mumps or experienced subclinical infections. The titres resulting from the vaccination were all lower than those of the two sera from persons convalescent from mumps but in some instances the discrepancy was not great. The failure of response to a second inoculation in individuals who had shown an effective response for the first is in conformity with experience with influenza virus vaccine (Beveridge, 1944).

DISCUSSION.

The discovery by Habel that mumps virus can be cultivated in the chick embryo has brought this virus into the group of viruses which can readily be handled in the laboratory. The most important of the possible developments is the production of an immunizing agent to protect people against the disease. Our preliminary experiments have shown that significant amounts of antibody can be produced with simple formalinized virus suspension and there is reason to hope that an effective immunizing agent may be developed.

The mumps virus shows certain similarities to the viruses of the influenza group which call for comment. With all these viruses red cells of a wide range of species including birds and mammals are agglutinated, the agglutinin is the virus itself, elution occurs and renders the cells insusceptible to further agglutination by the same virus and some others of the group. These features are the more significant since no haemagglutination has been shown by several other viruses tested in this and other laboratories except with the mammalian pox group (vaccinia, variola and ectromelia). The haemagglutinin of vaccinia is a soluble substance distinct from the virus; a much narrower range of cells are agglutinated and elution does not occur (Burnet and Stone, 1946). Although the size of the mumps virus has not been accurately determined, the filtration experiments of Habel and the centrifugation experiments reported in this paper indicate that it is about the same as that of influenza virus. The manner of growth in the chick embryo is much the same with mumps and influenza viruses inasmuch as many different tissues are susceptible, virus is liberated into amniotic and allantoic fluids and foci are not readily produced on the chorioallantois. Further study may reveal a closer relationship between these viruses than has hitherto been suspected.

The inhibitory effect on haemagglutination caused by allantoic fluid is also met with influenza virus (Rawlinson, 1946), but it is not so marked as with mumps virus. The inhibitory effect of yolk-sac suspension and the relative insensitivity of cells from many fowls were probably responsible for our failure to obtain haemagglutination when we tested for it after first isolating the virus. This experience indicates two pitfalls to be avoided when a new virus is being investigated. We have found no way of destroying the inhibitory substance without destroying the agglutinin but it is of some help when testing weak virus suspensions containing inhibitor to use a weak suspension of fowl cells (0·25 p.c.) and to test several dilutions of virus.

SUMMARY.

Observations on haemagglutination by suspensions of mumps virus revealed that in all probability the virus itself is the agglutinating agent. In this and in other respects, mumps virus haemagglutination resembles influenza virus haemagglutination.

Allantoic fluid suspensions of yolk sac or chorioallantois and amniotic fluid from embryos more than 12 days old contain a substance which inhibits haemagglutination by weak virus suspensions.

Using antisera prepared in rabbits or human convalescent sera and a haemagglutination inhibition technique, strains of virus from three widely separated sources appeared identical.

Preliminary experiments on vaccination of human subjects showed that formalinized virus produced appreciable amounts of antibody after subcutaneous inoculation.

Acknowledgment. It is a pleasure to thank Dr. F. M. Burnet for much helpful advice with this work.

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THE BIOLOGICAL ACTIVITY OF A SUBSTANCE RESEMBLING GLIOTOXIN PRODUCED BY A STRAIN OF *ASPERGILLUS FUMIGATUS*

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WITH A CHEMICAL ANALYSIS

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(Accepted for publication 29th March, 1946.)

In a previous communication (Stanley 1944) some properties of an antibacterial agent, provisionally designated "aspergillin", were described. Further investigations indicate that it is similar to, if not identical with, gliotoxin—a nitrogenous sulphur-containing antibacterial substance produced by *Gliocladium fimbriatum* and *Trichoderma lignorum* (Weindling and Emerson, 1935; Dutcher, 1941; Johnson *et al.*, 1943, 1944; Bruce *et al.*, 1944; Dutcher *et al.*, 1944a and b). A substance resembling gliotoxin has been isolated also from *Aspergillus fumigatus* (Menzel *et al.*, 1944; Glister and Williams, 1944). Until more is known about both substances and an adequate comparison becomes possible, the active material isolated from *Aspergillus fumigatus* will be referred to as "aspergillin".

In view of the lack of information available on the biological activity of this antibacterial substance, the following investigations were carried out.

EXPERIMENTAL.

Production of Aspergillin.

The mould was isolated from agar and tellurite plates which had been incubated for several days at 37° C. in an atmosphere containing 3·5 p.c. CO_2. At this temperature the mould grew rapidly on Sabouraud's medium, and spored within 48 hours. At 24° C. a similar condition was reached after incubating for three days. As only small quantities of aspergillin were produced at the higher temperature, all cultures were grown at 24° C. The inoculum consisted of a suspension of spores prepared from seven-day cultures of the mould grown on Sabouraud's medium at 24° C. Five ml. of Czapek-Dox medium (Hobby *et al.*, 1942) were added to each slope and suspensions of spores prepared by rubbing the surface growth with a nichrome loop. These suspensions were pooled and 1 ml. added to each Roux bottle containing 150 ml. of Czapek-Dox medium. Under these conditions the maximum concentration of aspergillin was obtained by the fifth day, at which time the medium had reached a pH = 7·0.

There was little variation in production of active material. On the fourth day, however, the metabolic fluid of some bottles was deep bronze in colour, while that of others was pale yellow. The pigment, which varied in colour according to the pH of the medium, was precipitable with strong alkali as a flocculent reddish-purple deposit. It was soluble in acid and gave a pale yellow solution. On neutralization this changed to a bronze colour. It exhibited no antibacterial activity, but may be a derivative of fumigatin.

Estimation of Antibacterial Activity.

Two methods were used, viz. the ring test (Abraham *et al.*, 1941) and a serial dilution method. In the former test, double zones, uneven boundaries, errors in taking measurements and inaccuracies due to differing rates of diffusion may obscure the result. The results set out in Fig. 1 show that in the ring test, the size of the zone of inhibition depends, amongst other things, on the number of organisms in the inoculum. A standard solution of the sodium salt of penicillin (titre = 10^{-4} against *Staph. aureus*) was included for comparison. This was diluted to give an antibacterial titre (as determined by serial dilution in broth) of 1/40. A saline solution of aspergillin was diluted so that it possessed the same activity against *Staph. aureus* as the penicillin solution. In each ring test 0·5 ml. of these solutions was used.

Aspergillin is more sensitive to variation in the number of organisms present than is the sodium salt of penicillin. Though having the same antibacterial activity when tested by the serial

dilution method (inoculum = 10^{-3} organisms), they have an identical zone-size only when the inoculum contains a little more than 5×10^6 organisms.

This method was discarded as a quantitative test and was rarely used for the detection of aspergillin. A serial dilution method can be performed just as rapidly and more accurately providing the inoculum consists of a constant number of organisms (see Fig. 2), and the medium contains no substance capable of suppressing the action of the compound being titrated and supports maximal growth of the test organism (Atkinson and Stanley, 1943). The results recorded in Fig. 2 indicate, as did those obtained by the ring method, that the end titre of antibacterial activity of aspergillin varies with the number of organisms in the inoculum more than does that of penicillin. The antibacterial activity of monacrin (5 amino-acridine hydrochloride) was not subject to this variation.

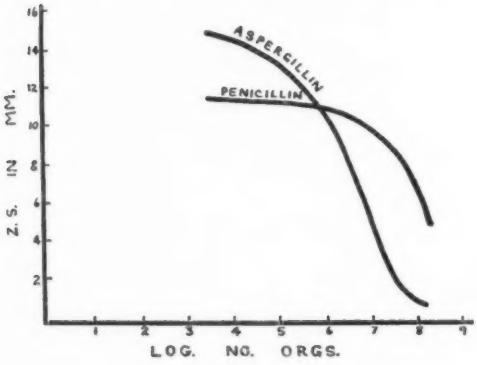

Fig. 1.

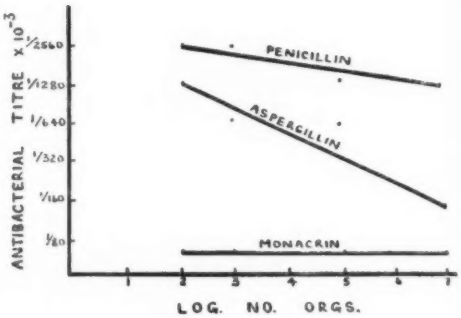

Fig. 2.

The technique of the serial dilution test adopted was essentially the same as that of Atkinson and Stanley (1943). An inoculum of 10^3 organisms in Ringer's solution was added with a dropping pipette (Wilson, 1922) to 5 ml. of serial double dilutions of aspergillin in the culture medium. The antibacterial titres of aspergillin determined in nine different media, using *Staph. aureus* as the test organism, were identical. Wright's broth was adopted as the standard test medium because of its wide application.

Purification.

All active material was readily removed from the metabolic fluid by shaking for one minute with an equal volume of ether which may be used for the extraction of three batches. With the pH between 6·5 and 8·0, almost all the inactive pigment remained in aqueous solution. One ml. of ethyl alcohol was added for every 100 ml. of extract, and the ether distilled off, leaving a sticky mass. This residue was dissolved in a minimum of boiling alcohol. The yellow alcoholic solution was placed in the cold overnight when aspergillin separated out as white needle-like crystals arranged in compact rosettes up to 5 mm. in diameter.

Occasionally it crystallized out as yellow needles. The average yield was 80 mg. from one litre of metabolic fluid.

Further purification was obtained by washing the crystals in cold alcohol and recrystallizing twice—once from ethyl acetate and once from alcohol. The melting point was between 172° C. and 180 ° C., with obvious decomposition. The method employed by Johnson *et al.* (1943) in the extraction of gliotoxin was also successful for the extraction of aspergillin.

In water aspergillin gives a neutral solution and is soluble on boiling to the extent of 0·5 mg./ml. When dissolved in boiling normal saline it gives a pale yellow solution which becomes colourless on cooling. The antibacterial activity is not destroyed by autoclaving for 30 minutes at 15 pounds pressure.

Activity in vitro.

Bacterial sensitivity. The antibacterial titre of aspergillin was determined against 16 bacterial species and one strain of yeast. Readings were observed every 24 hours for six days. Thus it was possible to estimate both the bacteriostatic and the bactericidal titres. The latter was confirmed by subculturing those tubes which did not show turbidity after the fourth day. On and after the fifth day subcultures were sterile.

Titrations against the anaerobes were incubated in an anaerobic jar with the necessary controls. The inoculum in the case of *Cl. tetani* consisted of one drop of a 24-hour culture. The yeast was isolated from the cerebro-spinal fluid of a fatal case of torulosis, and titrations against

this organism were carried out at 24° C. Additional tests were performed with *N. meningitidis* inoculated on to nutrient agar slopes containing 5 p.c. horse serum and 1 p.c. glucose and to which various concentrations of aspergillin were added. Each tube was inoculated with a loop from an 18-hour culture of the organism grown on the same medium, and incubated in the presence of 3·5 p.c. CO_2. A clearly defined end-point was obtained. No antibacterial activity was demonstrated against *Myco. tuberculosis*.

The results are shown in Table 1.

TABLE 1.

Organism.	Source.	Aspergillin dilutions × 10^{-4}. 1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/8192
C. diphtheriae gravis	throat swab	—	—	—	—	—	—	—	—	—	—	—	+	+	..
Strep. pneumoniae	sputum	—	—	—	—	—	—	—	—	—	—	—	+	+	..
C. diphtheriae gravis	throat swab	—	—	—	—	—	—	—	—	—	+	+	+	..	..
N. meningitidis	C.S.F.	—	—	—	—	—	—	—	—	—	—	—	+	..	..
C. diphtheriae mitis	throat swab	—	—	—	—	—	—	—	+	+	+	..	..	..	..
Strep. pneumoniae	sputum	—	—	—	—	—	—	—	—	+	+	..	..	..	..
Strep. pyogenes	blood culture	—	—	—	—	—	—	—	—	+	+	..	..	..	..
Viridans streptococci	blood culture	—	—	—	—	—	—	—	+	+	+	..	..	..	..
Cl. tetani	stock culture	—	—	—	—	—	—	—	+	+	..	..	..	..	..
Cl. welchii	stock culture	—	—	—	—	—	+	+	+	..	..	..	..	..	..
Br. abortus	Glenfield	—	—	—	—	—	—	+	+	..	..	..	..	..	..
Staph. aureus	blood culture	—	—	—	—	+	+	+	+	..	..	..	..	..	..
Staph. aureus	stock culture	—	—	—	—	+	+	+	..	..	..	..	..	..	..
B. subtilis	stock culture	—	—	—	—	—	+	+	..	..	..	..	..	..	..
Yeast	C.S.F.	—	—	—	—	—	+	+	..	..	..	..	..	..	..
Bact. flexneri	stock culture	—	—	—	—	—	+	..	..	..	..	..	..	..	..
S. typhi	faeces	—	—	+	+	+	..	..	..	..	..	..	..	..	..
S. typhi	stock culture	—	—	+	+	+	..	..	..	..	..	..	..	..	..
S. typhimurium	mice	—	+	+	+	+	..	..	..	..	..	..	..	..	..
Pr. vulgaris	pus	+	+	+	+	+	..	..	..	..	..	..	..	..	..
Ps. pyocyanea	pus from ear	+	+	+	+	+	..	..	..	..	..	..	..	..	..
H. influenzae	C.S.F.	+	+	+	+	+	..	..	..	..	..	..	..	..	..

— = bactericidal titre (no growth after 5 days).
+ = bacteriostatic titre (growth only *after* 24 hours).
.. = growth in 24 hours.

Aspergillin-resistant organisms. Resistant strains of *Staph. aureus, S. typhi* and *Cl. welchii* were obtained by making successive subcultures in media in which the concentration of aspergillin was increased at each subculture. After twelve subcultures over a period of six weeks, these organisms, for which the original titres were 1/640,000, 1/60,000 and 1/1,280,000, grew in broth containing aspergillin diluted 1/5,000, 1/2,500 and 1/5,000 respectively. There was no evidence of loss of this resistance at the end of three months and after many subcultures.

With *Staph. aureus* altered metabolism was indicated by a delay of 48 hours in the usual fermentation reactions. Altered respiration was indicated by the ability of the resistant strain to grow freely under anaerobic conditions. This was strikingly shown in shake cultures. Colonies of the resistant strain grew evenly throughout the medium (10 cm. deep), while growth of the original strain occurred only within 2 to 3 mm. of the surface. The resistant staphylococcus did not show decreased sensitivity to penicillin, but a penicillin-resistant strain of the original staphylococcus was more sensitive to the action of penicillin and monacrin (see Table 2).

TABLE 2.

Drug.	Staphylococci.	Dilutions of drug × 10^{-4}. 1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Aspergillin	Original	—	—	—	—	—	—	—	+	+	+	+
	Aspergillin-resistant	—	+	+	+	+	+	+	+	+	+	+
	Penicillin-resistant	—	—	—	—	—	—	—	—	—	+	+
Penicillin	Original	—	—	—	—	—	—	—	—	—	—	+
	Aspergillin-resistant	—	—	—	—	—	—	—	—	—	—	+
	Penicillin-resistant	—	—	—	—	—	+	+	+	+	+	+
Monacrin	Original	—	—	—	+	+	+	+	+	+	+	+
	Aspergillin-resistant	—	—	—	+	+	+	+	+	+	+	+
	Penicillin-resistant	—	—	—	—	—	+	+	+	+	+	+

+ = growth. — = no growth.

The resistant strains of *Staph. aureus* and *Cl. welchii* showed a marked loss of virulence which was not regained after subculture for three months.

The suppression of aspergillin activity. Various biological substances were tested for their ability to suppress aspergillin. Oxyhaemoglobin was the only one of these which completely suppressed its activity. Rabbit serum caused a slight reduction of activity. Aspergillin is not destroyed but only rendered inactive against bacteria as it can be recovered from the aspergillin-haemoglobin complex by acidification and extraction with ether. Denaturation of the globin by boiling did not free all the aspergillin. The remainder, however, was detected in the flocculent precipitate that formed on heating.

The possibility that the phenomenon was due to adsorption was explored as follows. Aspergillin (0·04 p.c. in saline) was mixed with an equal volume of horse serum, oxyhaemoglobin or saline, and the mixtures dialysed through cellophane for seven hours against two volumes of saline. The dialysates were titrated against *Staph. aureus*. In the presence of saline, one-half of the aspergillin passed through the membrane, in the presence of serum, one-quarter, and in the presence of oxyhaemoglobin, one-eighth.

The possibility that aspergillin was inactive in the oxidized form was suggested by the fact that oxyhaemoglobin suppressed its activity while carboxy-haemoglobin did not. For further evidence of the validity of this suggestion the actions of hydrogen peroxide and of potassium permanganate were tested. The aspergillin solution was mixed with an equal volume of hydrogen peroxide (3 p.c.). After standing for 15 to 30 minutes manganese dioxide was added, until oxygen ceased to be liberated, to destroy the remaining peroxide. After 15 minutes 88 p.c., and after 30 minutes 99·5 p.c. of the aspergillin was inactivated. An equal volume of 0·1 p.c. potassium permanganate completely inactivated the strongest aspergillin solution.

Activity in vivo.

Toxicity. No toxic effects were observed when 1·0 ml. of the strongest saline solution obtainable (0·04 p.c.) was administered subcutaneously into mice. This amount was injected in two equal volumes, one being given in the back and one in the belly. However, when the same volume of strong suspensions, made by boiling aspergillin in a mixture of water and alcohol until the alcohol was removed was given the mice died within 24 hours. Post-mortem examinations revealed that the lungs of all mice were a bright pink in colour. The prolonged steaming of saline solutions did not increase the toxicity.

No toxic effects were observed when 10 ml. of the saline solution were injected intravenously into rabbits. Rabbits were also injected intramuscularly and subcutaneously with 0·5 ml. of this solution. Forty-eight hours later tissue at the site of injection was removed and paraffin sections were cut. Histologically these showed little variation from the saline controls. Very slight leucocytic infiltration in the interfascicular spaces was observed. Until stronger aqueous solutions can be prepared a better determination of toxicity is not possible. Using the strong suspensions the M.L.D. for mice would be between 40 and 60 mg. per kg. body weight (the M.L.D. for gliotoxin was estimated at 50 mg. per kg. body weight).

Treatment of experimentally infected animals. Mouse protection tests were carried out with a strain of *Strep. pneumoniae*. The bacteriostatic titre of aspergillin against this strain was 10^{-1}. The bactericidal titre was 10^{-3}. Each mouse received 0·5 mg. of aspergillin injected subcutaneously in two equal volumes, 0·5 ml. being given in the back and 0·5 ml. in the belly. The inoculum (intraperitoneal) consisted of 0·1 ml. of an 18-hour serum-broth culture. Post-mortem examinations were conducted on all dead mice. *Strep. pneumoniae* was recovered from the heart-blood or liver of each mouse. Only one out of twelve animals survived.

Aspergillin has not been administered *per os* or by intravenous injection in experimentally infected animals.

CHEMICAL ANALYSIS DEMONSTRATING THE POSSIBLE IDENTITY OF ASPERGILLIN WITH GLIOTOXIN

By JOHN A. MILLS.

Physical properties. Aspergillin conformed closely to the physical properties recorded by Johnson *et al.* (1943). It was always obtained as long, flat, white or pale yellow needles, quite stable in the dry state. It was sparingly soluble in water, more soluble in dilute hydrochloric acid, and for other solvents its solubility increased in the order methanol, chloroform, pyridine. Methanol was used for recrystallization of various batches, and served to remove traces of insoluble gum present in some of the crude preparations. Solutions of aspergillin in chloroform were quite unstable to light, depositing an amorphous powder after one day.

None of the samples of aspergillin showed a sharp melting point, all passing slowly from the solid state to a brown syrupy liquid at about 180° C., with preliminary softening and yellowing from 170° C. The instantaneous decomposition temperature on an aluminium block was

215–220° C. (Johnson *et al.* report 221° C.). At lower temperatures aspergillin was more stable to heat, 30 minutes heating at 140° C. causing no appreciable change.

Optical activity[1] was determined using a 0·5 p.c. solution in chloroform. The average of twelve readings showed a specific rotation of $[\alpha]_D^{20^\circ} = -256$. This is in close agreement with that obtained for gliotoxin ($[\alpha]_D^{26^\circ} = -255 \pm 15$).

Analysis. Analysis of two separate batches of aspergillin gave the results: C, 48·42, 48·55; H, 4·47, 4·36; N, 8·38, 8·48; S, 19·06, 19·26. $C_{13}H_{14}N_2O_4S_2$ requires C, 47·85; H, 4·32; N, 8·59; S, 19·6. Methoxyl groups were absent. No suitable method was available for determining the molecular weight.

Chemical properties. The outstanding features of the chemistry of aspergillin were the same as those recorded for gliotoxin (cf. Bruce *et al.*, 1944; Dutcher *et al.*, 1944), viz. stability to acid, instability to alkali, and ready removal of sulphur as hydrogen sulphide.

Aspergillin could be recrystallized unchanged from hot, very dilute hydrochloric acid. On treatment with cold, dilute caustic soda solution part of the aspergillin went into solution, giving a pale yellow liquid, which on acidification after a few minutes, gave a flocculent precipitate and evolved the characteristic odour of hydrogen sulphide, the vapours blackening lead acetate paper. Lability to alkali with liberation of hydrogen sulphide is characteristic of β-keto-sulphides, such as cysteine (Nicolet, 1931), and aspergillin may be a compound of this type.

Aspergillin evolved hydrogen peroxide very rapidly when warmed with hydriodic acid.

Derivatives. Bruce *et al.* (*loc. cit.*) reported the preparation of well-defined di–benzoyl, di–*p*–nitrobenzoyl and di–*p*–bromobenzoyl derivatives when gliotoxin was treated with the appropriate acid chlorides in pyridine. Under the same conditions, aspergillin gave a di–benzoyl and a di–*p*–nitrobenzoyl derivative, each comparable in solubility and melting point to the corresponding derivative of gliotoxin.

Dibenzoyl-aspergillin, made from 50 mg. of aspergillin, was recrystallized three times from chloroform-methanol mixture, to give white pearly scales, m.p. 193–6° C. decomp. (Bruce *et al.*, report 192–3° C.). (Found: C, 60·94; H, 4·18. $C_{27}H_{22}N_2O_6S_2$ requires C, 60·70; H, 4·12).

Di–p–nitrobenzoyl–aspergillin, made from 33 mg. aspergillin, and also recrystallized from chloroform-methanol mixture, was obtained as brownish nodular masses of minute nedles, m.p. 187–90° C. decomp. (Bruce *et al.*, 189° C.). (Found: C, 51·97; H, 3·56. $C_{27}H_{20}N_4O_{10}S_2$ requires C, 51·90; H, 3·25).

DISCUSSION.

From the evidence presented in this paper it seems reasonable to assume that gliotoxin and aspergillin are identical. Chemical analyses agree very closely with those obtained by Dutcher and his colleagues.

The possible mode of action of aspergillin is revealed by the investigations on suppressors and resistant organisms. The susceptibility of aspergillin to inactivation by oxyhaemoglobin, hydrogen peroxide and potassium permanganate, but not by carboxy-haemoglobin, and the striking alteration of respiratory activity shown by resistant staphylococci suggest that aspergillin may act by interfering with the respiration of these organisms.

Aspergillin-resistant strains of *Cl. welchii* and *Staph. aureus* were shown to be avirulent. If this is universal, then aspergillin assumes importance from the point of view of therapy. There is, therefore, a similarity to penicillin, as it was shown (Rake *et al.*, 1944) that a marked decrease in virulence for mice was associated with development of bacterial resistance to penicillin. Both penicillin and aspergillin are bacteriostatic for Gram-positive organisms at a dilution of several millions, and organisms resistant to both germicides are avirulent. But the fact that an aspergillin-resistant organism is not resistant also to the action of penicillin indicates some difference in their mode of action. According to recent work on "drug-resistant" bacteria (McIntosh and Selbie, 1943) this offers a new line of approach to the study of the mode of action of antibacterial substances. The aspergillin-resistant staphylococcus did not vary in its sensitivity to the other germicides (see Table 2). The penicillin-resistant staphylococcus, however, showed a fourfold increase in sensitivity to aspergillin and monacrin (see McKee, *et al.*, 1944).

[1] Determined by N.F.S.

It is, as yet, too early to estimate the chemotherapeutic value of aspergillin. Solutions capable of protecting mice have not been obtained. Attempts to overcome these difficulties merit further investigation as aspergillin is acid-resistant and heat-stable—desirable properties in any chemotherapeutic agent.

Addenda.

Since this paper was prepared for publication more information concerning gliotoxin and aspergillin has come to hand.

Dutcher *et al.* (1945), have proposed provisional structural formulae for gliotoxin and desthiogliotoxin. Their evidence suggests that the most probable formula for gliotoxin is a rather novel one.

Dutcher *et al.* (1945), point out that the inactivation of gliotoxin by cysteine observed by Cavallito and Bailey (1944) probably resulted from the alkali used in preparing the test solution. Furthermore, no chemical reaction could be demonstrated between gliotoxin and thioglycollic acid until alkali was added to the mixture. This is in agreement with the observation that the antibacterial activity of aspergillin was not suppressed in the presence of cysteine hydrochloride.

Aspergillin has been used successfully in an oil base in the local treatment of infectious dermatitis due to *Staph. aureus.*

SUMMARY.

The preparation and the biological and chemical properties of aspergillin, an antibacterial substance produced by *Aspergillus fumigatus*, have been described.

Evidence, such as melting point, solubility, antibacterial activity, M.L.D. for mice, specific rotation and chemistry of derivatives is presented, which indicates that aspergillin is similar to, if not identical with, gliotoxin ($C_{13}H_{14}N_2O_4S_2$).

Some idea of the mode of action of aspergillin has been deduced from a study of the nature of aspergillin-resistant organisms and of substances capable of suppressing its antibacterial activity.

Acknowledgment. I am indebted to Dr. A. E. Platt for his continued help throughout the course of these investigations.

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STUDIES ON THE CULTIVATION *IN VITRO* OF NORMAL AND PATHOLOGICAL GUINEA-PIG BONE MARROW

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(Accepted for publication, 11th March, 1946.)

The object of this paper is to record tissue culture observations simultaneously carried out with haematological investigations on the guinea-pig, which were reported previously, (Brandon Fastier, 1945).

Cultivation of human bone marrow with a view to the elucidation of cell maturation problems was first attempted by Osgood and Brownlee (1937a). This method involved the use of vaccine vials and was later replaced (Osgood and Brownlee, 1937b) by one using a complicated apparatus allowing for the continuous irrigation of marrow cells. Further publications by these workers as well as by Israels (1940) showed a reversion to the simplified vial technique. From the work of Osgood and Brownlee (1937a and 1937b) many points on the physiology of bone marrow cells in culture have been recorded, probably the most important being the wide variations in conditions necessary for growth. Any oxygen tension between 3 and 40 p.c. may be employed, pH may vary from 7 to 8, osmotic pressure from that of 0·1 to 1·0 p.c., sodium chloride and temperature from 0 to 41° C. Cytological studies gave the life of the polymorphonuclear neutrophil as 48 to 49 hours, the eosinophil 8 to 28 days and the basophil 12 to 15 days. Cultures of lymphocytes proceeded to multiply amitotically as long as the experiment proceeded, while monocytes and histiocytes were the result of rapid promonocyte proliferation. Rachmilewitz and Rosin (1943), employing a different technique involving rabbit bone marrow implanted in coagulated serum, studied the problem from a cell relationship aspect but unfortunately gave no differential counts of individual cells.

In order to correlate the previous paper (Brandon Fastier, 1945) and the present communication the pertinent facts from the former work may be given. Dietary conditions influence the erythropoietic activity of the marrow. Post-haemorrhagic conditions show marked hyperplasia with medullary erosion in the bone marrow. The marrow is normally in a normoblastic state and shows no evidence of megaloblastosis.

Marrow cultivation from normal animals as well as from the above pathological states forms the concluding section of this investigation.

EXPERIMENTAL.

Two methods of cultivation were employed. The first was a modified version of the continuous irrigation apparatus of Osgood and Brownlee (1937b) and the other the vaccine vial technique of the same authors (1937a) and Israels (1940). In both cases the medium consisted of a balanced saline having the following components:

Sodium chloride	8·00 gm.
Potassium chloride	0·37 „
Sodium bicarbonate	0·23 „
Calcium chloride (anhydrous)	0·17 „
Magnesium chloride (hydrous)	0·21 „
Disodium hydrogen phosphate	0·15 „
Potassium dihydrogen phosphate	0·03 „
Magnesium sulphate (hydrous)	0·07 „
Dextrose	1·00 „
Distilled water	1,000·0 ml.

In order to determine whether homologous serum was necessary for active growth two sets of cultures were prepared, one containing 35 p.c. guinea-pig serum in the above saline, and the other 35 p.c. human serum from which the haemagglutinins had been absorbed by the appropriate red cells. The serum/saline mixtures were Seitz filtered under low pressure and stored after sterility testing in 50 ml. batches at 4° C.

Bone marrow was obtained from the animal immediately after death by aseptic transverse section of the long bones with gentle aspiration of the contents with a sterile pipette. The combined marrows from each animal were transferred to a sterile centrifuge tube together with 7 ml. of the balanced saline, gently agitated to break up cell clumps and slowly centrifuged. The buffy

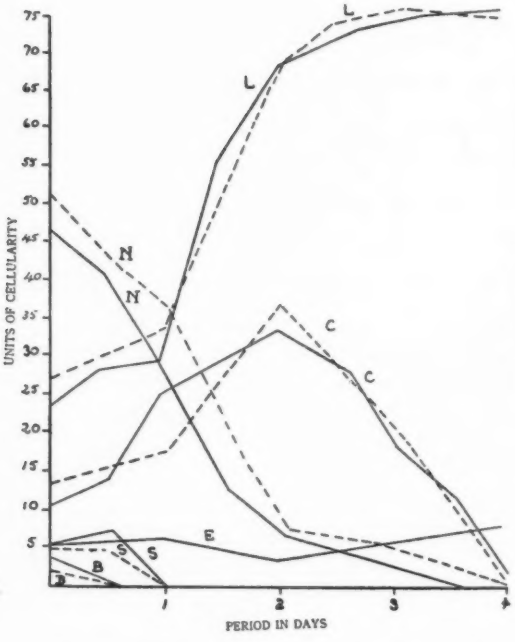

Fig. 1. Duplicate marrow cultures in guinea-pig serum (continuous line) and human serum (interrupted line). Lymphocytes (L); Neutrophils (N); Eosinophils (E); "Stabs" (S); Type B Normoblasts (B); Type C Normoblasts (C).

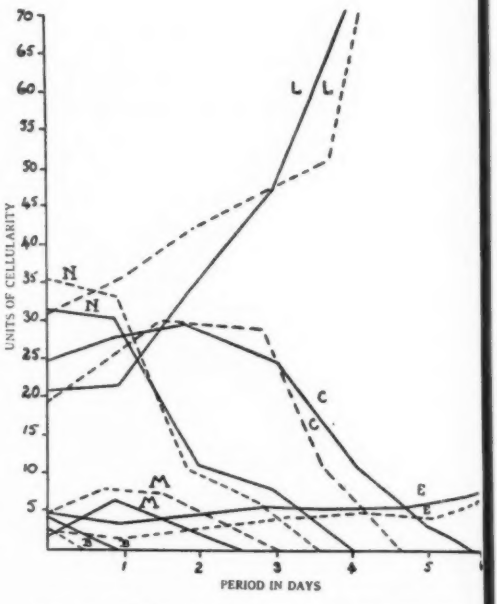

Fig. 2. Cultivation of two bone marrows from animals on bran-enriched diets. (Legend similar to Fig. 1. Monocytes (M)).

coat was removed and suspended in the serum/saline to give a haemocytometer count of 10,000 cells per c.mm. According to the culture technique employed the standardized cell suspension was introduced into either the main culture flask of the continuous irrigation apparatus or divided into 3 ml. portions for vial cultures. Cytological studies were made on the culture prior to incubation at 37° C. and at 12 hour intervals. Smears were stained by Leishman's method together with supravital staining with Janus green and neutral red. Vaccine vial cultures (total volume 5 ml. containing 3 ml. fluid) were aerated by drawing filtered air through the contents every 14 hours.

(In order to avoid repetition of long Tables, bone marrow differential counts on classes of animals from which cultures were prepared are referred to as Tables 1–4 of the author's previous paper, Brandon Fastier, 1945.)

The bone marrows from several animals considered normal from a haematological standpoint (Table 1, 1945) were used for cultivation in both guinea-pig and absorbed human serum media. Duration of culture was from periods of six to ten days after which cells appeared vacuolated and nuclear absorption of supravital stains indicated degeneracy and death. The results were, on the whole, in general agreement as shown by two representative cultures plotted in Fig. 1. From these

graphs it will be seen that polymorphonuclear leucocytes remain nearly stationary in numbers for a period of one day, apparently maturation of metamyelocytes, etc., maintaining an equilibrium with polymorph destruction. After the first day polymorph counts begin to drop until only degenerate forms remain in the culture on the fourth day. Lymphocytes remain fairly constant over the first day but proceed to multiply amitotically and rise steeply in numbers to a peak on the fourth day after which they decline gently in number. Type B normoblasts owing to their initially low level were not in appreciable numbers on the first day but their undoubted descendants, i.e. type C normoblasts, showed a marked rise in numbers to about the second day after which they remained constant over a day and gradually declined to elimination. Eosinophils experienced a decrease in numbers over the first day but after two days' culture regained their former level and were present when the culture was discarded.

Bone marrows from animals with bran-enriched diet (Table 2, 1945) were cultivated in duplicate serum mixtures with the same close approximations. Cultivation yielded short-lived cells owing to the large percentage of mature segmental neutrophils and relatively few immature cells. These results are expressed in Fig. 2, from which it will be seen that the neutrophil series declines sharply to extinction on the fourth day as maturation of the low percentage of "stab" polymorphonuclear leucocytes is unable to maintain equilibrium with destruction of the mature segmental forms. Normoblasts of type C show the same decline as their precursors and are not able to gain maturity in sufficient numbers to influence the cell count. Monocytes show a slight increase on the first day but decline to elimination on the third day. Lymphocytes exhibit their previous ability to multiply amitotically and reach their maximum when the culture was discarded on the fourth day. In the above series of cultures mitotic figures were comparatively rare and were not observed in cells of the granulocyte series more mature than the myelocyte and never in the lymphocyte series.

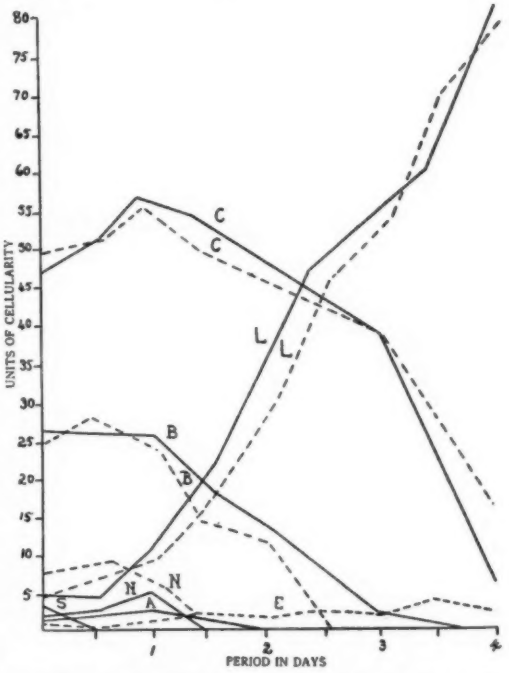

Fig. 3. Cultivation of bone marrows from two animals following haemorrhage. (Legend similar to Fig. 1. Type A Normoblasts (A)).

Animals suffering from acute haemorrhage show a typically active bone marrow (Table 4, 1945) and it is not surprising that cultures from such guinea-pigs are predominantly erythroblastic. Observations on these cultures (Fig. 3) were unhampered by effete products of leucocyte degeneration owing to the low level of the latter cells in the marrow. It is probably due to this fact that the life of such cultures is of longer duration than others. From the results of this grouping of animals we see the same typical replacement curves for the normoblastic series which, owing to the large numbers of cells initially present, are of greater magnitude than those experienced previously.

In all cases supravital staining and observations on motility were used to determine cell viability but in cases such as those of the lymphoid elements which are non motile and show few stainable granules this criterion was of no use. The above facts complicated the study of the Foa-Kurloff cell *in vitro*. As has been mentioned (Brandon Fastier, 1945) this cell is lymphoid or monocytoid in nature and contains inclusion bodies (this term is one of convenience and is not to be interpreted as showing affinities with those found in virus infections) which appear to undergo cyclical changes. Cultivation of marrow and blood containing these cells was short lived and resulted in their disappearance from the culture after 13 hours. At first the inclusion rapidly absorbs neutral red thus offering a ready method of counting these cells. Later this property is lost and after six hours the recognition of these cells by supravital stains is impossible.

A comparison of the two techniques employed may be conveniently summed up under the two headings: (a) simplicity and (b) accuracy. In both cases the vaccine vial is superior to the cumbersome continuous irrigation method. Not only is the former free of connecting tubes which overlie the culture flask and make sampling difficult but also in the vial method sterility is easily maintained and if contamination does occur only one of the duplicate cultures is lost. Both methods give reproducible results and on cross-duplication experiments it would seem that neither has any greater degree of accuracy over the other. For biochemical investigations the vial technique is to be preferred owing to the relatively small bulk of medium necessary to maintain cell viability and therefore not over-diluting any compounds requiring micro-estimations.

SUMMARY.

The results of cultivation *in vitro* of normal and pathological bone marrow from guinea-pigs have been recorded. In all cases myeloid elements are eliminated on about the third day of culture while lymphoid cells multiply amitotically to their maxima on the fourth to sixth day. Throughout all cultivation periods the same conditions of growth are observable; the viability of a strain of differentiated cells is dependent on the relative numbers of their immediate precursors in the culture. This work confirms the length of life of the polymorphonuclear neutrophil as being two days (Osgood and Brownlee, 1937b), but destruction curves of these cells are dependent on the numbers of myeloid and "stab" forms present and their relative maturation rates. In a comparison of the two methods of culture the technique which has been most successful is that employing a vaccine vial as the culture flask. This method is to be recommended for cytological and biochemical investigations on cultures of bone marrow.

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THE EFFECT OF MORPHINE ON DYE CONCENTRATION CURVES FOR PLASMA VOLUME DETERMINATION IN DOGS

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(*Accepted for publication 15th April, 1946.*)

During some work involving plasma volume estimations in dogs by the dye method, the results of an investigation on the effect of various drugs on the accuracy of such determinations in man were published (Bowler *et al*, 1944). It appeared that the use of morphine could introduce serious errors and as this drug had been used in all our animals for pre-anaesthetic sedation, an investigation of its effect was desirable.

Bowler *et al.* gave patients about to undergo major operations an injection of dye before operation. The dye concentration curve was determined throughout the operation. A second injection of dye was given and the concentration curve again determined. About half the dye concentration curves so obtained were described by the authors as "anomalous". In all cases a morphine–hyoscine–atropine mixture had been administered. The effects of the second injection of dye, the type and severity of operation and of the anaesthetic were examined. Following on this work a study was made on twenty normal volunteers. The results showed that in man the injection of morphine sulphate produced an effect on the dye concentration curve, which persisted in some cases for as long as five hours after administration of the drug. The authors suggest that the effect is due to uneven distribution of the dye in the circulation and to abnormally rapid elimination.

Bonnycastle (1942) stated that the effect of morphine sulphate in dogs is to increase the total blood volume by some 20 p.c. while the plasma volume remains constant. Hence the drug must cause the introduction of large volumes of red blood corpuscles into the circulation and the author considers that this is due to contraction of the spleen. The quantity of cells necessary to increase the blood volume of a 20 kg. dog (the average size of dog used) by 20 p.c. can be calculated using the average normal blood volume per kg. of 79 c.c. Courtice (1943). This value, together with the information in Table 1 of Bonnycastle, show that the spleen must provide at least 300 c.c. of cells. This is most unlikely and hence his results with morphine sulphate must be treated with reserve.

Since a study of the available literature left us in doubt as to the effect of morphine in dogs it was decided to investigate this question.

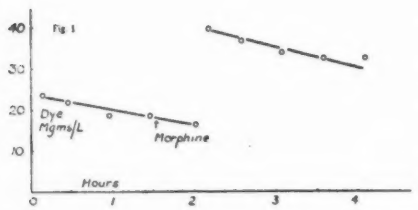
METHODS.

Mongrel dogs of both sexes with weights ranging between 5 and 13·5 kg. were used. They were fasted overnight. Chlorbutol intraperitoneally was used as the anaesthetic, approximately 1 c.c. per kg. of 40 p.c. solution in 40 p.c. alcohol being required. In all experiments the following routine was observed: A 5 c.c. sample of blood was taken from the carotid artery into a small glass pot. Three c.c. were measured into a centrifuge tube containing 0·6 c.c. of isotonic sodium oxalate solution (1·6 p.c.) and well mixed. Eight milligrams of T–1824 (Eastman Kodak Company) per kg. of body weight, were then injected into the external jugular vein. The same 0·2 p.c. solution was used throughout the experiments. After an interval of 6 minutes, a second sample of 5 c.c. blood for the estimation of plasma volume was withdrawn and measured as described for the previous sample. Three further samples were taken similarly at intervals of 30, 60 and 90 minutes after the dye injection. Immediately following the 90 minute sample, one grain of morphine sulphate was injected subcutaneously. One hundred and twenty minutes after the dye injection, another sample was taken for the estimation of the residual dye concentration prior to a second plasma volume determination. A similar injection of T–1824 was now given and blood samples taken 6, 30, 60, 90 and 120 minutes after the second dye injection.

The total cell volume was determined on each sample as diluted with the anticoagulant. The capillary tube method of Guest and Siler (1933) was used. The tubes were centrifuged for 30 minutes at 4,000 r.p.m. in a head giving a mean radius of 13 cm. The results were corrected for the dilution by the anticoagulant solution. The remaining samples were centrifuged and the plasma withdrawn for dye estimations.

Dye Estimations.

All dye estimations were made using a photoelectric colorimeter similar in design but not identical with that described by Kennedy and Millikan (1938). A layer depth of 2·5 mm. was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Dye
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Morphine
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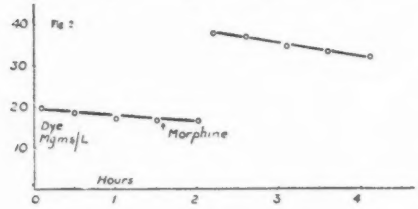
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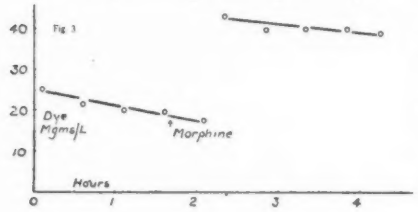
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Dye
Mgms/L
Morphine
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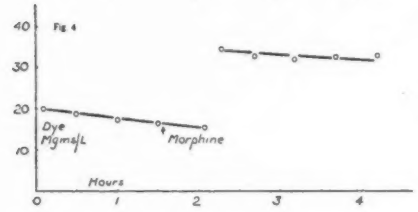
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Dye
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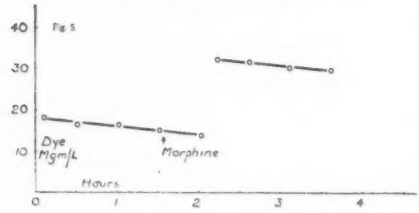
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Dye
Mgm/L
Morphine
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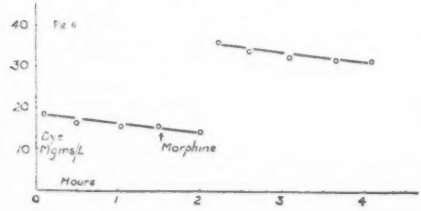
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Dye
Mgms/L
Morphine
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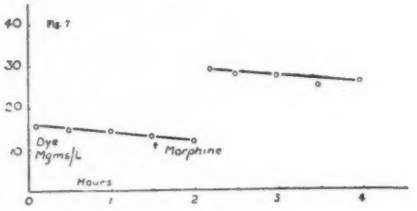
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Dye
Mgms/L
Morphine
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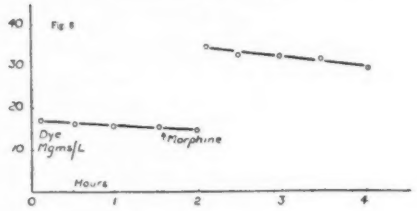
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Dye
Mgms/L
Morphine
Hours
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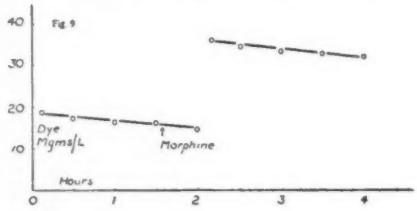
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Dye
Mgms/L
Morphine
Hours
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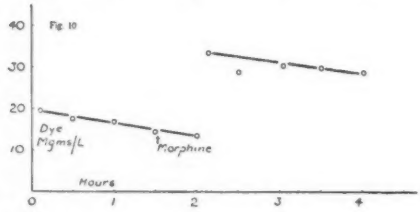
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Dye
Mgms/L
Morphine
Hours
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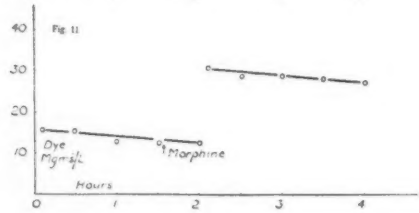
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10
Dye
Mgms/L
Morphine
Hours
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1
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3
4

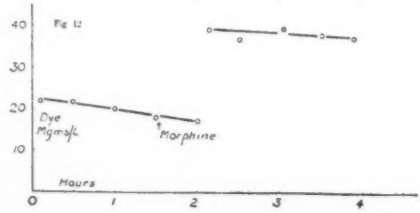
Fig. 12
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30
20
10
Dye
Mgms/L
Morphine
Hours
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4

used throughout. A standardization curve relating light absorption and dye concentration was constructed for the sample of dye used. The plasma from the initial blood sample was used to obtain the setting for 100 p.c. transmission. The percentage light absorption for each of the remaining samples in turn, was determined against this setting, which was checked between each sample measurement. The appropriate dye concentrations were read from the standardization curve.

Calculation of Results.

These followed the usual pattern, allowance being made for the dilution of plasma by the anticoagulant solution. The values obtained from the samples taken immediately following the dye injections were used for the calculation of plasma volume. In the case of the second determination allowance was made for the residual dye left in circulation. The value for this adjustment was obtained as previously mentioned. Where dye concentration values are stated they have been corrected to give the value in the circulating plasma.

RESULTS.

In Figs. 1 to 12 are given the curves showing the dye concentration before and after the injection of morphine. The relevant values for plasma volume, total blood volume, etc., are also shown. The figures in brackets after the second value for the blood volume are the estimated quantities of blood withdrawn between the first and the second injections of dye. It will be observed that when allowance is made for these withdrawals the values agree, except in one

EXPLANATION OF FIGURES.

Fig. 1.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	175	51·5	361	193	5
2 hours	175	49·4	345 (34)	170	

Fig. 2.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	364	51·3	747	383	9·1
2 hours	339	54	737 (27)	398	

Fig. 3.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	430	51·5	886	456	13·6
2 hours	427	50·7	866 (31)	439	

Fig. 4.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	317	50·6	642	325	7·7
2 hours	474	41·6	813 (32)	339	

Fig. 5.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	309	42·2	534	225	7
2 hours	316	44·3	565 (29)	249	

Fig. 6.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	429	41·1	728	299	10·4
2 hours	378	42·5	657 (34)	279	

Fig. 7.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	594	41·3	1,012	418	11·8
2 hours	556	40·6	930 (28)	374	

Fig. 8.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	555	50·2	1,114	559	11·8
2 hours	470	54·4	1,003 (25)	533	

Fig 9.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	338	49·5	656	318	7·7
2 hours	294	51·7	608 (22)	314	

Fig. 10.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	268	45·4	489	221	6·8
2 hours	264	46·8	496 (24)	232	

Fig. 11.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	451	38·4	731	280	9
2 hours	394	39·0	646 (25)	252	

Fig. 12.

	P.V.	Ht.	B.V.	Total R.C.V.	Wt.
Initial	292	49·8	581	289	7·7
2 hours	287	52·8	608 (27)	321	

Key.

P.V.	Plasma volume (c.c.).
Ht.	Cell volume per cent.
B.V.	Blood volume (c.c.).
Total R.C.V.	Total red cell volume (c.c.).
Wt.	Weight (kilogrammes).

instance, within the experimental error of the method. One animal also showed considerable haemodilution, the cause of which is obscure. The curves show that there is no disturbance produced by morphine. Table 1, giving the mean disappearance of dye in mg. per litre per hour, shows that there is no signicant difference in the rates before and after the drug.

Contrary to the view expressed by Bowler *et al.* (1943), it is of interest to note that the rate of disappearance of the dye was independent of its concentration in the plasma.

TABLE 1.

Mean dye disappearance rates.

Before morphine mg./l/hour.	After morphine. mg./l/hour.	Difference.
3·8	3·6	− 0·2
1·7	2·9	+ 1·2
3·8	2·2	− 1·6
2·3	0·8	− 1·5
2·2	2·0	− 0·2
2·3	2·6	+ 0·3
2·2	1·8	− 0·4
1·3	2·8	+ 1·5
1·5	1·6	+ 0·1
2·2	2·2	0·0
3·3	3·1	− 0·2
1·9	2·0	+ 0·1
2·5	1·2	− 1·3
	mean	− 0·17

$t = 0{\cdot}67$ hence P = approx. 0·5.

No increase in the *total cell volume* of the order of that mentioned by Bonnycastle (1942) has been found by us. The absence of anomalies of the type found in man may be due to the very great difference between the human and canine tolerances for morphine.

SUMMARY.

An investigation has been made of the blood volume and dye disappearance rates of T-1824 before and after the injection of morphine. No effect has been found.

Acknowledgments. The authors wish to acknowledge their indebtedness to the Director, Dr. A. B. Corkill, for his advice and criticism and to Mr. Edgar Rouse, Chairman of Directors, Kodak (Australasia) Pty. Ltd. for the supply of the dye used.

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AN ELECTRON MICROSCOPE INVESTIGATION OF THE CUTICLE OF WOOL

by E. H. MERCER AND A. L. G. REES

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(Accepted for publication 15th March, 1946.)

The cortex of keratin fibres, which is the basis of their remarkable elastic properties, has been made the subject of many chemical and physical investigations (Astbury and Woods, 1933 and Woods, 1938); the cuticle on the other hand, presents greater difficulties in investigation and consequently has been relatively little studied. The cuticle, which forms a sleeve of flattened imbricated cells enveloping the cortex, nevertheless fulfils several important functions: its chemical inertness and physical toughness serve to protect the more vulnerable cortex, it is a limiting factor in the processes of absorption, and it confers upon the fibre the property of unsymmetrical friction, the primary cause of wool felting.

We do not wish to review here the optical microscopy of the wool fibre, but some information is necessary for the understanding of the electron micrographs (E.M.G.s.), and it will be advantageous to discuss this briefly before proceeding. A comprehensive account may be found in Frölich, Spöttel and Tänzer (1929).

The cortex of the fibre is composed of thin spindle-shaped cells about 100μ long cemented together to form an optically homogeneous filament. The cuticle cells are flat, roughly oval-shaped bodies about 30μ in diameter and of thickness ranging between 0·5 and 1·5μ. Physical and chemical evidence suggest that the cortex is fibrous and that the cuticle possesses no molecular orientation.

The histology of the hair follicle, within which the wool fibre is developed, has been frequently described. (See, for instance, Kronacher and Lodemann, 1930). As a first approximation, the fibre may be regarded as a rod of keratin extruded from the follicle as a result of the pressure developed by the continuous proliferation of cells. The cells are scarcely distinguishable from one another at the level of the papilla. The marked changes in shape are to be ascribed partly to the effects of the physical and chemical environment of the follicle and partly to original differences in cell composition. The proto-cortical cells of the non-medullated wool fibre occupy the centre of the follicle and are elongated by extrusion through the narrowing follicular neck. The proto-cuticle cells on the other hand are hindered in their movement by the proximity of the follicle "walls" and are flattened and wrapped around the body of the cortex. The basic organizational difference between the two types of cells, the fibrous cortical and non-fibrous cuticle, are to be referred back to the occurrence or absence of an orientating flow at this level in the follicle. Certain other minor structural details of the cuticle cell may also be referred to the moulding effect of the follicle; these will be discussed later.

During the pre-keratinous stage at which the fibre components are being moulded into shape the protein they contain is soft and plastic. The subsequent hardening process or keratinization sets the cells in the shape they present when finally isolated from the fibre by the action of agents which attack preferentially the intercellular cementing material.

The results of the electron microscope investigation described in this paper, together with evidence drawn from other sources, provide a detailed structural picture of the cuticle cell or scale. Electron diffraction patterns obtained from several components of the fibre, including the scales, are similar, indicating features of molecular structure common to all. Some observations on the effect of shrinkage-reduction treatments on the scales will also be described.

ELECTRON MICROSCOPY OF WOOL.

Studies of wool using the electron microscope (E.M.) have already appeared (Zahn, 1943, and Hock and McMurdie, 1943); and these have confirmed conclusions concerning fibre structure drawn from other sources. We have used methods similar to these authors to resolve fibres into fragments suitable for microscopy. A brief account of our own work has also appeared, (Mercer and Rees, 1946).

The wool used in our experiments was a fine (70's quality) Australian merino. Such wool consists of cuticle and cortex only. It was degreased by several extractions with petroleum ether, washed with water containing about 0·1 p.c. of a sulphonated fatty alcohol and finally with distilled water. Although our observations are limited to this class of material, there is ample evidence of the similarity of the cuticle in other wool and hairs to suggest that the structural conclusions apply generally to the keratin fibres, (Rudall, 1941). Physical, chemical and enzymic means have been employed by previous workers to disintegrate the fibre into small fragments; we have found that the appearance of the scales in the E.M. depends on the method of isolation, and that only the physical methods such as scraping and grinding produce cellular material substantially in an original and unaltered form. The following were the techniques employed:

(i) *Grinding* was carried out in an ordinary ball mill using dry wool. The fibres break down suddenly after several days' treatment into a powder from which samples containing fragments of either cortical or cuticle cells can be obtained by fractional sedimentation.

(ii) By *scraping* a fibre or a small staple across the edge of a razor blade small samples of cuticle could be obtained. These samples were substantially free from corticular fragments if the scraping motion was against the scales and under appropriate pressure.

(iii) *Wool retted with trypsin* according to the method of Burgess (1934) (0·2 p.c. trypsin buffered with phosphates to pH 8·3 at 40° C.), breaks down in a few days into the component cortical and cuticle cells. The flat cuticle cells can be partially separated from the long thin cortical cells by fractional sedimentation.

Gentle grinding of the cellular fragments produced by the above methods between pieces of roughened glass usually yielded finer fragments more suitable for electron microscopy. The disintegrated fragments were dispersed in distilled water and a drop of the suspension allowed to dry on a Parlodion film mounted in the usual way for electron microscopy.

An examination of both cortex and cuticle has been made, but this publication is mainly concerned with the cuticle.

The microscope used was an R.C.A. Type EMU.

DESCRIPTION OF THE ELECTRON MICROGRAPHS.

Whole Cuticle Cells and Fragments.

Cells obtained by scraping and by grinding (Fig. 1) show little evidence of internal structure. The small differences in transmission giving an appearance of ill-defined light and dark areas may be ascribed to differences in total thickness, although the accumulation of electrostatic charge makes observation of any detail impossible. Profile photographs (Fig. 8), however, show that the surface is relatively smooth.

Cells isolated by the action of trypsin (Fig. 2) present an appearance characteristically different from those obtained by scraping. The smooth surface is replaced by a clearly defined variable, pitted and even honey-combed structure. Hock and McMurdie (1943) and Zahn (1943) have reproduced micrographs of damaged material evidently derived from structures similar to that of Fig. 2. The pores or vesicles range in diameter from a few hundred up to 2,000Å (0·2μ) in diameter and are often arranged in roughly parallel rows. Ridges and furrows in the surface also appear to run parallel to the pores. The cells preserve the above appearance even after prolonged retting in enzymic solutions.

The orientation of relatively large-scale features such as pores, ridges and furrows in the surface of cuticle cell remnants does not extend as far as the molecule, as is shown by the breakup phenomena illustrated in Fig. 3. Fracture occurs in no preferred direction. This is in marked contrast to the fibrous character of the cortical cells made apparent by striation and fission of fibrils down to 100Å diameter. E.M.G.s. of corticular fragments showing such features have already been published by the authors previously referred to, and others will be given in a further publication by us.

That the scale is a flattened cell is demonstrated clearly by Fig. 4 in which portion of the upper face of the cell has been broken off by grinding. The removed portion of this surface is still visible, and the crumpled interior surface of the lower face has been exposed.

Granular material (cell contents) has been observed in association with cells ruptured by grinding. A mass of such material still lying within the cell cavity may be seen in Fig. 4. A further E.M.G. of such cell contents is given in Fig. 5. These granules may be remnants of the nucleus, whose presence has been established in cuticle cells by special techniques (Kronacher and Lodemann, 1930; Hock, Ramsey and Harris, 1941). They are joined together in strings which are occasionally branched. This material may be responsible for the osmotic swelling of the cuticle cell (when attacked by chlorine water) known as Allwörden's reaction.

Thin web-like sheets have been occasionally observed in cellular material released by trypsin (Fig. 6). This structure is very thin but clearly defined, the resolution of its details is particularly good. Definite evidence of its rôle was not obtained, but it is not unlikely that it is a remnant of a cell membrane. Zahn (1943) mentions a "net-like membrane" perhaps similar to Fig. 6.

Fig. 1. Fragment of cuticle cell obtained from wool fibre by scraping.

Scalar fragments from wools superficially damaged by keratinolytic agents such as: sodium sulphide, caustic alkalis, halogens, and sulphuryl chloride, used to produce a so-called "unshrinkable finish", present a mottled uneven appearance all similar to Fig. 7. .The manner and degree of treatment approximated to that usual in commercial processes and need not be detailed here. The outlines of the structure made more clearly visible by enzymic action (Fig. 2) can be seen in Fig. 7. At the edge of the scale small excrescences often give evidence of an internal reaction leading to swelling, rupture and escape of decomposed material. The same attack at the scale edge can be seen in profile E.M.G.s. such as Fig. 8.

Profiles of Whole Wool Fibre.

The imbrications of the flattened cuticular scales are visible in the optical microscope but are on the limits of resolving power. The use of material as large as whole fibre in the E.M. is complicated by electrostatic charging, with consequent movement and image distortion; but, by the use of a very fine merino wool (80's quality) stretched over the usual mesh without a Parlodion

film and low beam currents, it was possible to obtain satisfactory results. Although the profile E.M.G.s. given here are not highly magnified the high resolving power of the E.M. enables the finest detail at the scale edge to be seen. Typical edges are reproduced in Fig. 8 (a and b) to show the manner in which the flattened cell overlaps its neighbour. The existence of a re-entrant angle is important.

Fig. 8 (c, d and e) show how a "shrink-proof" treatment (sulphuryl chloride) attacks the exposed edge of the scale preferentially, rounding it off, fraying it, and very probably rendering the edge particularly susceptible to further attack when subsequently exposed to alkaline

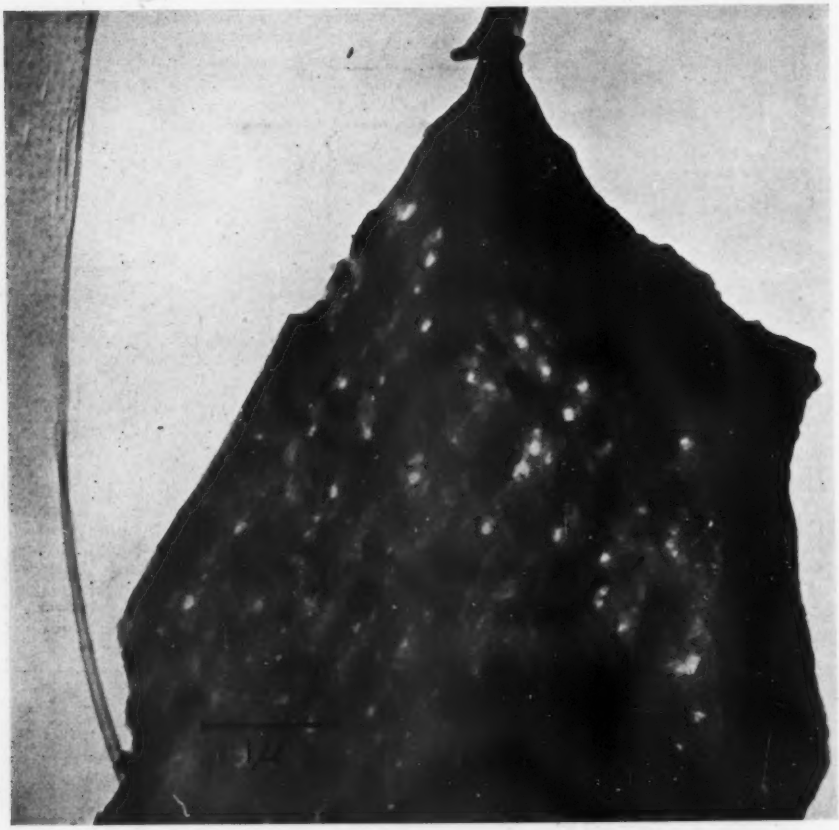

Fig. 2. Remnant structure, k_2, of cuticle cell prepared by the action of trypsin on wool fibre.

liquids. The appearance of scale edges attacked by other shrinkage-reduction treatments is similar, and not markedly dependent on the type of reagent used; chlorine- and bromine-treated fibres after treatment with mildly alkaline solutions (pH 10–11) show the most marked changes.

ELECTRON DIFFRACTION BY KERATIN PREPARATIONS.

Athough there have been many X-ray diffraction studies of keratin fibres (Astbury and Street, 1931; MacArthur, 1945), little has been attempted with electrons. Matano (1939) showed that films obtained by drying solutions of wool in sodium sulphide yielded a diffraction pattern. We have repeated this work on such films and are in agreement with Matano's figures. In addition to this, we have found that diffuse diffraction patterns can be obtained from several other components of the fibre. The same basic pattern of three diffuse rings was obtained from each of the following preparations:

(1) Solutions of wool in 1 p.c. sodium sulphide. About 80 p.c. of the wool dissolved. The residue was cuticle and fibrillar remnants. The solution was dialysed for 3 days in a thin cellophane bag. The dialysate was an opalescent solution yielding a white precipitate with mercuric chloride. A drop of this solution was dried to form a fragmentary film on a fine mesh for the diffraction tests.

(2) The resistant component of scales (see Fig. 2) made by separating the scale remnants from cortical cells by the fractional sedimentation of a suspension of the cells from enzyme-retted wool. The scales were washed free from salts, suspended in water and a drop dried on a fine wire mesh.

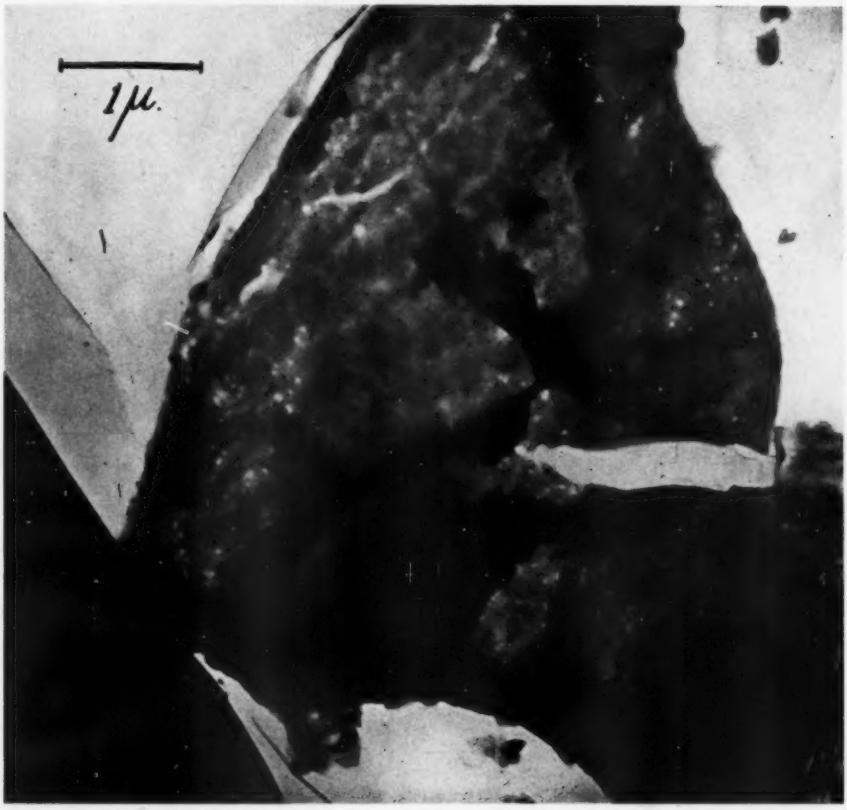

Fig. 3. Fragment of cuticle cell isolated from wool by trypsin showing effects of mechanical damage.

(3) Unoriented cortical cells separated from scalar fragments as in (2) and mounted in the same way.

(4) A solution, which was predominantly the more soluble component of the scales and intercellular cement, was prepared by treating wool with 0·5 M thioglycollic acid and 10 M urea adjusted to pH 8 with sodium carbonate. The wool was treated for 14 days at 40° C. and then allowed to stand for 3 months at room temperature. At the end of this time it was found that partial disintegration of the fibres had occurred and that cells could be obtained after shaking the wool with glass beads in a small mill for 2 days. The undissolved wool was removed and the solution dialysed for 5 days. Portion of this solution was dried on a fine wire mesh. The fibres and cells, which were removed, did not appear optically to be severely damaged.

The spacings calculated from the three rings yielded by each of these preparations are given in Table 1.

Fig. 4. Ruptured cuticle cell isolated by trypsin.

TABLE 1.

Spacings found in the electron diffraction patterns of preparations from wool.

Preparation.	Spacings in Å.		
Film prepared from solution of wool dissolved in Na_2S	1·07	1·92	4·28
Ditto (Matano, 1939)	1·11	1·97	4·26
Cuticular resistant component (k_2)	—	2·10	4·14
Solution of k_1 and intercellular cement in thioglycollic acid	1·06	1·96	4·32
Cortical cells	1·11	2·02	4·30

INTERPRETATION OF THE ELECTRON MICROGRAPHS.

Profile E.M.G.s. of intact fibres (Fig. 8) and E.M.G.s. such as Fig. 1 of cells obtained by scraping show that the undamaged cuticle presents a comparatively smooth featureless external surface. A comparison of Figs. 1 and 2 shows at once that the trypsin has removed a superficial layer and exposed a resistant

underlying structure. The precision with which the enzyme etches the resistant surface free from the less resistant material, and the fact that the scale is thereafter unaltered by weeks of exposure to the enzyme shows that there are two clearly defined components. For convenience in what follows the component digested by trypsin will be referred to as k_1 and the resistant component as k_2.

Fig. 5. Cell contents released by rupture of scales.

This nomenclature implies that we regard k_1 and k_2 as sub-varieties of the class of keratins; but we do so cautiously, recognizing that little is known of the chemical nature of the two components. We have not been able to obtain micrographs to show whether k_1 also covers the inside surface of k_2. The relation the two layers bear to each other and the general structure of the cell are shown diagrammatically in Fig. 9. The roughly parallel folds in the surface of k_2 probably arise from crumpling of the cell as it is forced up the follicle against the resistance of the adjacent inner root-sheath. This implies that the elements of the k_2 structure are present in the cells prior to keratinization.

On retting whole fibre with trypsin not only is k_1 etched away from k_2, but the intercellular cement holding the cuticle to the cortex and the cortical cells themselves together, is removed also, and apparently with similar facility. If we now regard the degree of digestibility by trypsin as a means of distinguishing and identifying the varieties of keratin, we must conclude a close similarity and probable identity between k_1 and the intercellular cement of the cortex.

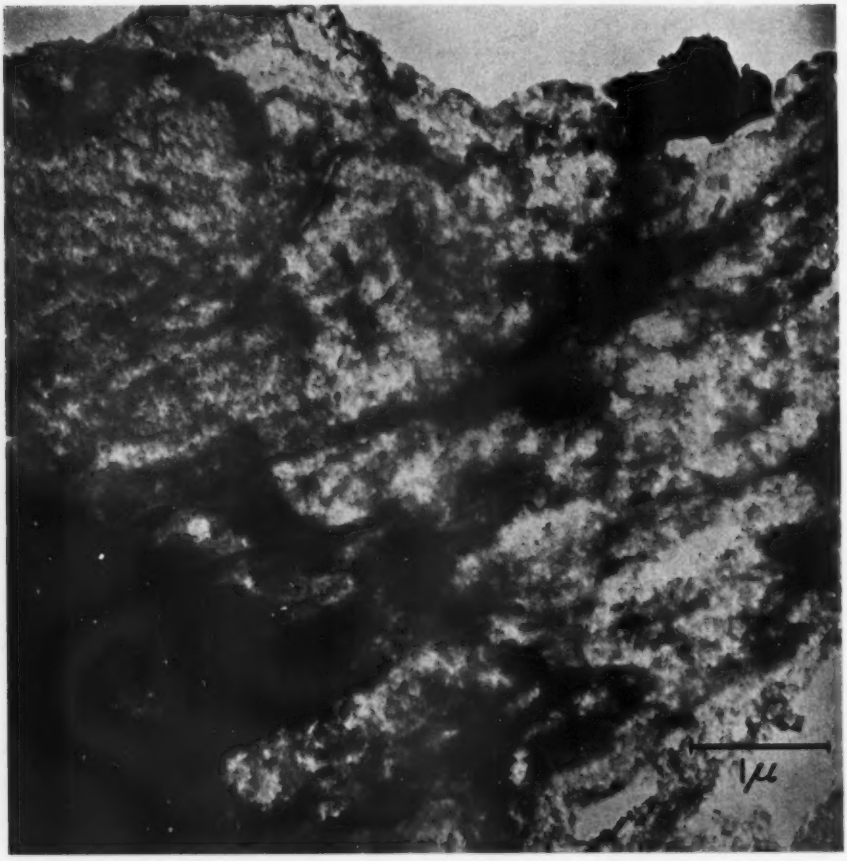

Fig. 6. Thin web-like sheet probably associated with scales. Released by trypsin from wool fibre.

Woods (1938), on the basis of optical and elastic evidence, has advanced the view that the intercellular keratin is continuous with a similar phase within the cortical cell. In this sense the cortical cell is a region within a continuous matrix of less crystalline keratin reinforced by fibrils of a further variety of more crystalline and resistant keratin. The appearance of these fibrils was described by Von Nathusius (1898) and their arrangement within the cell by Mercer (1942). In the course of our E.M. examination of the cortex we have

found evidence (to be published later) in support of Wood's view. It is plausible then to assume that the tryptic digestible k_1 of the scales is physically continuous and probably chemically identical with the matrix of the cortex. We differ, however, from Woods in regarding this corticular matrix as amorphous and sharply distinct from the crystalline fibrils.

Fig. 7. Example of chemical damage to cuticular scale. Specimen isolated by scraping from wool treated with sodium sulphide.

The different chemical and physical properties exhibited by k_1, k_2 and the corticular fibrils may be attributed to such factors as chemical composition, degree of polymerization or physical organization. Since no chemical analysis has yet been carried out on a single protein species from the keratin complex, it is difficult to draw definite conclusions. The figures for the whole fibre refer to a mixture of at least four keratinous components and small amounts of non-keratins. Geiger's (1944) analysis of a scalar material isolated by the use of pepsin probably refers to the component k_2, which on his figures differs considerably from whole fibre.

Evidence that, in spite of some chemical differences, there are some features of molecular structure common to all the components of the fibre was obtained by means of electron diffraction. Table 1 shows that all preparations examined contain predominantly the same molecular spacings. An analysis of the results will be attempted at a later date when further results are to hand; for the present we use them merely to indicate the basic similarity of the several varieties of keratin.

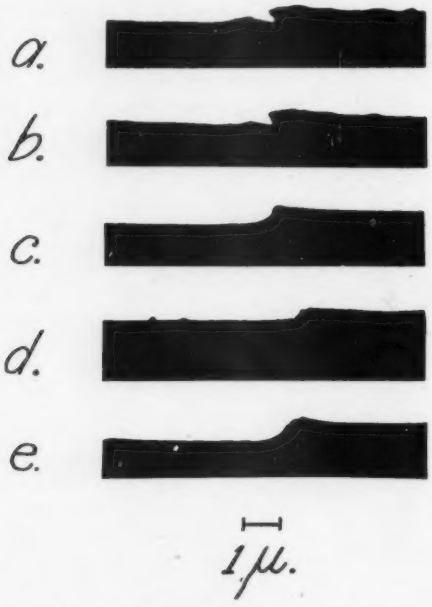

Fig. 8. Profile E.M.G.s. of cuticle of fibres. (Fibre in Black).
(a) and (b). Profiles of intact scale. (c), (d) and (e). Profiles of scale from fibre treated with sulphuryl chloride.

The possibility that all the samples contain a common component which is the diffracting material must be considered. The possible common component is the intercellular cement k_1. However, the E.M.G.s. of the resistant component show that k_1 has been almost completely removed by the trypsin. On the other hand the E.M.G.s. of corticular fragments show that the intercellular cement has not been entirely removed and therefore the diffraction may arise from these remnants, especially since the cement envelops the fibrils. The solution in sodium sulphide will contain derivatives from several components.

THE ACTION OF SHRINKAGE-REDUCTION TREATMENTS ON THE CUTICLE.

The cuticle confers upon the individual fibres of wool and hair the property of unsymmetrical friction. That is, the fibres are rougher in the tip to root direction than in the root to tip. Felting, which is the cause of the shrinkage of woollen garments, has been shown by Arnold (1929) and by Speakman and his colleagues (1931, 1933) to be due to a persistent rootward migration of each

individual fibre leading finally to a uniformly tangled and compact mass of interlacing fibres. The cause of the migration is the unsymmetrical friction and the rate of felting can be modified by altering the difference in friction. (Mercer, 1945, and Speakman, Chamberlain and Menkart, 1945). In general any chemical or physical treatment, which leads to superficial damage to the cuticle, seems to yield a felting-reduction or "unshrinkable" process.

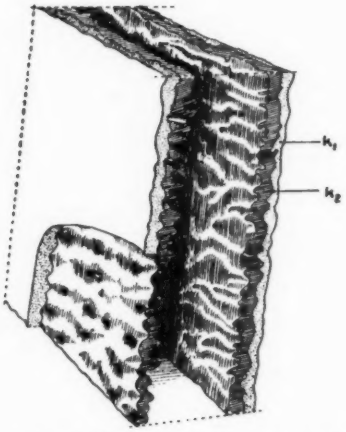

Fig. 9. Relation of the components k_1 and k_2 of the cuticle cell. The thickness of the cell is about 1μ. The width of the cell cavity has been exaggerated.

The cause of the difference in friction is usually held to be the imbrications of the scales, but some peculiarities of the property have led to other suggestions (Martin, 1944). In particular, the frictional difference can be reduced to a low value by shrinkage reduction treatments without altering the appearance of the scales in the optical microscope. In an attempt to clarify this situation we have compared electron-microscopically cuticular scales scraped from untreated and treated fibres, and also the cuticular profiles of these fibres.

Under the E.M., cells scraped from treated fibres showed some variety of appearance even when obtained from the same fibre. The incidence of the attack varies from scale to scale. In the case of each of the treatments the underlying k_2 structure was rendered more visible showing that the the reagent had attacked preferentially and partially etched away the k_1 component. Fig. 7 shows a scale scraped from a fibre treated with sodium sulphide. The nature of the attack resembles that of trypsin (which also yields an unshrinkable process), but the chemical reagents do not discriminate so precisely between the two components k_1 and k_2. Occasionally specific signs of decomposition at the scale edge can be found as in Fig. 7.

A better picture of the way in which the frictional properties are affected by these reagents can be obtained from E.M.G.s. of the cuticular profile. Fig. 8 shows the ratchet-like edge of the cuticle in untreated wool. The way in which one flat cell overlies the next is clearly visible. The fact that the scales are not everywhere sealed down to the next underlying scale, but possess a considerable overlapping unattached edge, is of interest in the explanation of the existence of unsymmetrical friction in the case when the fibre is rubbed against smooth hard surfaces in which the unevennesses are of a much smaller order than the scale edges, e.g. polished glass. A mechanical ratchet effect is not possible here. Rudall (Speakman and Menkart, 1945) has prepared a rubber model, which exhibits frictional difference against glass, from pieces of overlapping rubber arranged in a manner similar to Fig. 8(a).

Typical profiles of the same wool after treatment (Fig. 8c, d, e) show clearly that the projecting free edge is the seat of the initial attack. The sharp edge is often rounded off, or gives other signs of erosion, and the overlap has disappeared. It should be emphasized that the E.M.G.s were obtained from fibres which had not been rubbed after treatment. We could expect the frictional difference caused by such eroded edges to be reduced partly on account of the impairment of the ratchet effect and partly because the free edge has been removed or sealed over with decomposition products. Most washing takes place

in alkaline media pH 10–11 and it is possible that in addition to the damage initially produced, the degraded protein at the scale edges will be further attacked, removing more of the edge or developing a lubricating layer of gelatinous protein. This was confirmed for brominated fibres, as has already been done using chlorinated fibres and the optical microscope. (Speakman and Goodings, 1926).

SUMMARY.

The cuticular scale cell of wool contains two main components, k_1 and k_2, both probably sub-varieties of keratin, which are distinguished by their digestibilities in trypsin.

The superficial component k_1 forms a smooth surfaced layer covering the resistant component k_2 and is removed by the action of trypsin. k_1 is probably identical with the intercellular keratin, which binds the fibre, cuticle and cortical cells together.

The resistant component, which is not digested by prolonged exposure to trypsin, forms a honey-combed structure with characteristic pores and ridges and valleys arranged roughly linearly and parallel.

Chemical reagents and enzymes attack k_1 preferentially, exposing the surface of k_2 more or less clearly.

Shrinkage-reduction processes in particular attack the cuticle cell initially at the k_1 component of the exposed over-lapping edge of the scales and in this way reduce the "frictional difference" of the fibre surface.

Similar electron diffraction patterns are yielded by k_2, films of k_1 and intercellular cement, films derived from solution of whole fibre in sodium sulphide, and by isolated cortical cells.

Acknowledgments. We wish to thank Miss H. P. Meggs for preparing the drawing for Fig. 9, and Dr. H. E. Dadswell for permission to use the photographic facilities of the Division of Forest Products.

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